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FUNCTIONAL IDENTIFICATION AND MODELING OF NERVES IN AIRWAYS

By

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A DISSERTATION

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ABSTRACT

Constriction of mammalian airways is primarily driven by nerves that release the neurotransmitter acetylcholine (ACh). One FDA approved class of drugs blocks ACh-induced constriction in asthma. However, nerves contain other neurotransmitters that may also be targets for asthma treatment. To further characterize the role of various neurotransmitters in modulating airway constriction, nerves in isolated mouse airways were activated using electrical field stimulation (EFS) which causes release of neurotransmitters. Constriction and relaxation caused by neurotransmitters were pharmacologically blocked to decipher their roles in airway function. EFS caused an ACh-mediated constriction phase and a relaxation phase blocked by capsaicin and indomethacin.

Drug distribution models build outward from first principles using computational methods to describe the movement of drugs in an organism. These models create a rigorous mathematical framework to allow for simulation of in vitro and in vivo processes. My hypothesis was that a computational drug distribution model can consolidate experimental data into a mathematical description of the functional role of nerves in mouse airways. Data from isolated trachea experiments informed the design of compartment-based computational models. By minimizing the sum of squares error, the computational models predicted kinetic parameters that described ACh-induced trachea constriction in response to EFS.

Identifying the mediators of the relaxation phase that were eliminated by capsaicin and indomethacin was the second necessary step toward development of a complete functional model. Sensory neuropeptides and prostanoid receptors were
pharmacologically blocked to decipher their roles in regulating airway relaxation. Results of experiments indicated that PGE$_2$ is a candidate mediator of the EFS-induced relaxation phase. Therefore, the PGE$_2$ receptor EP$_2$ was incorporated into the drug distribution model. The updated Multiple Neurotransmitter model was able to reproduce biphasic EFS responses, consistent with experimental observations.

This work contributes a novel perspective to the study of the functional neurotransmitter systems in the lung. Coupled with \textit{in vitro} and \textit{in vivo} experiments, a more complete model may aid \textit{in silico} testing of drugs, furthering the development of new therapeutics for the treatment of asthma and other lung diseases.
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CHAPTER 1: ASTHMA

Introduction

Asthma is a chronic disease of the airways characterized by both excessive airway constriction and airway hyper-responsiveness leading to reduced airway diameter and limited airflow. Inflammation, airways smooth muscle (ASM) structural remodeling, increased mucus secretion, and enhanced bronchoconstriction lead to shortness of breath, wheezing, and coughing. An asthma diagnosis is defined by the symptoms, not the underlying pathology. Some pathologies are refractory, leaving gaps in treatment options for some patients. These patients continue to suffer, and frequent emergency department visits contribute to the high financial burden of the disease. New therapies may fill the treatment gaps leading to increased quality of life for asthma patients.

Epidemiology

The World Health Organization estimates that 235 million people have asthma worldwide. The incidence is increasing in Westernized countries. Globally, 250,000 people die from asthma every year. In the United States 18.9 million people have asthma (8.2% of the population), of which 7.1 million are children. Males are more likely to develop asthma as children, but females make up the majority of cases in adulthood. The US Centers for Disease Control and Prevention report that about 50% of all asthma cases in the US are uncontrolled (CDC 2014).

Pathology

Asthma can broadly be broken into atopic asthma and non-atopic asthma. Atopic asthma is characterized by type 2 allergic airway inflammation and these patients make up the majority of cases. In atopic asthma, the patient is exposed to an allergen, which
activates T helper (Th2 type) cells. These cells in turn release interleukin-4 (IL-4) and interleukin-5 (IL-5). IL-4 activates B cells to differentiate and to produce IgE antibodies against the allergen. The IgE antibodies bind to receptors on the surface of mast cells and upon re-exposure to the inciting allergen, cause the mast cells to degranulate. Degranulation causes the release of histamine, prostanoids, leukotrienes, serotonin, and other inflammatory mediators. These chemical mediators attract eosinophils and neutrophils into the lungs. Patients with atopic asthma often have elevated blood and sputum concentrations of eosinophils. IL-5 released from T helper cells further activates eosinophils, causing the release of catalytic proteins that damage the epithelium, leading to more inflammation (Rosenberg et al., 2013). Inflammation increases the permeability of the lung epithelium, allowing outflow of these mediators into the ASM and interstitial space where they can activate sensory and parasympathetic nerves. Locally, parasympathetic nerves release acetylcholine causing bronchoconstriction.

Between 5 and 10% of asthmatic patients have some form of severe asthma. These subtypes of asthma are refractory to traditional treatments. These patients are often treated with high doses of systemic corticosteroids, leading to undesirable systemic side effects. Furthermore, patients with severe asthma are more likely to visit the emergency department because of symptoms, and they contribute a majority of asthma-related health care spending (Yim and Koumbourlis 2012). The National Institutes of Health have highlighted the importance of treating severe asthma by creating ten Severe Asthma Research Program (SARP) sites around the country (National Heart Lung and Blood Institute 2018). Their mission is to characterize the phenotypes of the different severe asthma subpopulations.
Steroid-resistant asthma is one subtype of severe asthma. Patients with steroid-resistant asthma, as the name suggests, do not see an improvement of symptoms after treatment with steroids. Some of these patients have a Th2 inflammatory phenotype yet still do not respond to steroid treatment. Other patients with severe asthma have a low level of Th2 inflammation yet have an asthmatic phenotype (Peters et al. 2018).

Paucigranulocytic asthma, asthma without increased levels of granulocytes, must have another mechanism of causing airway hyper-responsiveness besides Th2 inflammation. Patients with little airway inflammation instead display hypertrophy of ASM and airway remodeling (Agache et al. 2012). One potential cause of smooth muscle growth is enhanced parasympathetic nerve activation (Scott and Fryer 2012). While atopic asthma is primarily immune-driven, blockade of parasympathetic nerves in the airways has also been shown to decrease eosinophil infiltration into the lung in a mouse model of allergic airway hyper-responsiveness (Damera et al. 2010). Overall, nerves are an attractive target for the treatment of both atopic and severe forms of asthma.

**Drugs Used to Treat Asthma**

Despite the large number of patients with uncontrolled asthma, there are many treatments for the disease. However, their lack of efficacy in steroid-resistant and other severe asthma subpopulations necessitates novel therapeutics. The current standard therapies for the treatment of asthma are briefly reviewed here.

**β2 Adrenergic Receptor Agonists**

β2 adrenergic receptors (β2ARs) present on ASM are G-protein coupled receptors (GPCR) that signal via Gαs. Activation of the receptors by β2 agonists leads to an increase in intracellular cAMP which in turn activates protein kinase A (PKA). PKA
phosphorylates a variety of proteins downstream including myosin light chain kinase. Phosphorylation of myosin light chain kinase inactivates it, leading to a decrease in myosin phosphorylation and, ultimately, resulting in relaxation of ASM. Short-acting beta agonists (SABAs) work acutely by binding directly to β₂ARs and reversing enhanced airway constriction during asthma attacks. Albuterol (salbutamol) is the most commonly used SABA. While it can be administered orally, it is most often administered directly to the lungs via a metered-dose inhaler (Proventil®, ProAir®, and others). Long-acting beta agonists (LABAs) are administered daily to prevent asthma attacks. The longer half-lives of these drugs allow them to remain at the site of action in the lungs longer than the SABAs. Formoterol and salmeterol are two common LABAs. LABAs are always combined with an inhaled corticosteroid for the treatment of asthma and are only used alone to treat COPD. In 2013 the FDA approved vilanterol, a once-daily “ultra”-long-acting beta agonist. Soon after olodaterol and other once-daily β₂ agonists were approved to control asthma and chronic obstructive pulmonary disease (COPD) symptoms.

**Theophylline**

Theophylline (Bronkadyl®) is a caffeine-like methylxanthine that does not enter the central nervous system at therapeutic doses. It acts by blocking the breakdown of cAMP in ASM cells by inhibiting the activity of the enzyme phosphodiesterase. Like with β₂ agonists, increased cAMP leads to decreased myosin phosphorylation and increased ASM relaxation. Its side effects, similar to caffeine overdose, include anxiety, restlessness and insomnia limiting, its clinical utility.
**Corticosteroids**

Corticosteroids act to decrease the body’s immune response. Their wide ranging effects are mediated by binding to intracellular glucocorticoid receptors and regulating gene transcription. While the mechanisms of action of corticosteroids are complex, two prominent roles in the airways are to decrease synthesis and release of inflammatory mediators and to decrease infiltration and activity of inflammatory cells. Administering corticosteroids directly to the airways (inhaled corticosteroids (ICS)) reduces the incidence of severe systemic adverse effects. However, patients with refractory asthma may take high doses of corticosteroids orally to manage symptoms. Adverse effects associated with steroid therapy include hypertension, diabetes, weight gain, decreased wound healing, and increased risk of infection. ICS are taken daily, often in combination with a $\beta_2$ agonist or antimuscarinic, to prevent asthma attacks.

**Leukotriene Receptor Antagonists**

Montelukast (Singulair®) and Zafirlukast (Accolate®) block the cysteinyl leukotriene receptor 1 (CYSLTR1). By blocking CYSLTR1, leukotriene receptor antagonists (LTRA) reduce the binding of the inflammatory leukotrienes LTC$_4$ and LTD$_4$. These leukotrienes cause both bronchoconstriction and inflammation, both of which can be prevented withLTRAs. While they are not first-line therapies, LTRAs can be useful in treating atopic asthma as well as allergic rhinitis. A related class of drugs, the leukotriene synthesis inhibitors, block 5-lipoxygenase, the enzyme that generates leukotrienes. Zileuton (Zyflo CR®) is the most used drug in this class, however its twice-daily dosing makes it less popular than the once-daily LTRA montelukast.
Antibodies

Several monoclonal antibodies have been approved for the treatment of allergic asthma. Omalizumab (Xolair®) is a monoclonal antibody that targets a patient’s own IgE antibodies. By binding to and inactivating IgE antibodies produced by B cells, omalizumab limits the ability of allergens to trigger the release of inflammatory mediators that can worsen asthma symptoms. It is used in severe atopic asthma that cannot be controlled by ICS.

Another group of monoclonal antibodies that were approved by the FDA in 2015 target interleukin-5 (IL-5). Reslizumab (Cinqair®) and Mepolizumab (Nucala®) both inactivate IL-5, a key cytokine released from T helper cells that recruits eosinophils into the lung. They are indicated for patients with severe atopic asthma that have an eosinophilic phenotype (blood eosinophil count > 400/µL).

Antimuscarinics

Atropine is a derivative from the deadly nightshade plant (Atropa Belladona) and is a non-selective antagonist of muscarinic receptors. Muscarinic receptors on ASM cells are the primary target for the parasympathetic neurotransmitter acetylcholine (ACh) and their activation causes profound bronchoconstriction in the lungs. While atropine has not been prominently used for the treatment of airway diseases, it remains a useful pharmacological tool for studying cholinergic innervation in isolated airways. Derivatives of atropine, however, are currently used to treat airway disease. Ipratropium is an atropine derivative that is used to relax the airways during acute exacerbations in COPD and asthma. Tiotropium (Spiriva®) is another antimuscarinic with a longer duration of action. It was originally approved to prevent exacerbations in COPD, but in 2014 was
approved as an add on medication for the treatment of asthma. Aclidinium (Tuodorza®) and umeclidinium (Incruse®) are two newer antimuscarinic drugs with longer biologic half-lives approved for the treatment of COPD. Certain populations may be more sensitive to antimuscarinics than others. In patients with uncontrolled asthma, there was a benefit found in adding tiotropium to ICS and LABA therapy (Kerstjens et al. 2012). Currently, the antimuscarinics are the only class of drugs that target the nerves in the airways to treat asthma.

**Lung Innervation**

Mammalian lungs are innervated by several diverse nerve populations. Autonomic and sensory nerves release a variety of neurotransmitters to control airway diameter by causing the airways to constrict or to relax. In addition to acute effects, these neurotransmitters can promote morphological changes in the tissues of the airways. However, the populations of nerves present, and the types of neurotransmitters released, in human lungs and in animal models of human lung disease are poorly understood. Understanding the neurotransmitter systems causing nerve-mediated constriction and/or dilatation of mouse airways may support the use of mouse models to study the functional role of nerves in lung disease. Furthermore, elucidating these mechanisms may identify novel therapeutic targets on autonomic or sensory nerves for treatment of asthma and other lung diseases.

The pulmonary nerves can broadly be divided into two groups, the autonomic nerves and the sensory nerves. Autonomic nerves can be further divided into parasympathetic and sympathetic nerves.
**Parasympathetic Nerves**

Parasympathetic innervation of the lung is primarily branches from the vagal nuclei of the medulla in the brainstem. Parasympathetic signals are transmitted along the vagus nerve to the heart, lungs, and inferior organs (Belvisi 2012). The primary neurotransmitter released from parasympathetic nerves is ACh. Released ACh can act at two types of ACh receptors, nicotinic and muscarinic receptors. Muscarinic receptors are a family of 5 GPCRs (M1-5). M1, M3, M5 receptors predominantly signal via Gq G protein complex while M2 and M4 principally signal via to Gi. M3 is the dominant constrictive muscarinic receptor subtype present on ASM. Activation of this GPCR by ACh leads to dissociation of Gαq from the receptor which binds and activates phospholipase C (PLC). PLC cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) into diacylgerlycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 diffuses through the cytoplasm to the sarcoplasmic reticulum where it activates Ca2+ channels. The released Ca2+ binds to calmodulin which in turn can bind to and activate myosin light chain kinase. Phosphorylation of myosin by myosin light chain kinase leads to ASM constriction.

M2 receptors are also present on the ASM in some species. Upon activation the Gαi protein dissociates and inhibits cAMP production. While this does not appear to directly cause ASM contraction, it may be important in inhibiting relaxation caused by any Gαs-coupled GPCR agonists (such as β2 agonists) (A. Fryer and Jacoby 1998). Also relevant to this study are the M2 receptors present on nerves in the neuromuscular junction. These receptors act as a negative-feedback regulator by inhibiting the release of ACh. Also regulating the concentration of ACh in the neuromuscular junction is the enzyme acetylcholinesterase (AChE). AChE cleaves ACh into acetic acid and choline,
terminating its biologic activity. Nerve agents (e.g. sarin, VX) and some pesticides (e.g. parathion, malathion) inhibit AChE, causing ACh-induced excitotoxicity which can be deadly. Together AChE and presynaptic M₂ receptors tightly regulate the ACh concentration in the neuromuscular junction.

Nerve density appears to be homogenous in the upper airways. Balentova, Conwell, and Myers (2013) found no difference in the density of immunohistochemical staining with the neuron-specific marker PGP9.5 between the superior trachea, the inferior trachea, or the primary bronchi. While nerve density may be homogenous, the types of nerves and their distribution is not well characterized. Furthermore, receptor density varies throughout the lung. The greatest density of muscarinic receptors is in the inferior trachea. The density of muscarinic receptors decreases deeper in the lungs (Fryer and Jacoby 1998).

A subpopulation of parasympathetic nerves does not release ACh. These non-adrenergic, non-cholinergic (NANC) nerves are parasympathetic in origin and structure. Two of the most prominent NANC nerve neurotransmitters in the lungs are nitric oxide (NO) and vasoactive intestinal peptide (VIP). Acutely, NO acts as a direct bronchodilator by increasing intracellular levels of cGMP. In patients with COPD, NO is measured as a biomarker of inflammation. However, a majority of NO in the lungs is generated by inducible nitric oxide synthase (iNOS) in the epithelium rather than neuronal nitric oxide synthase (nNOS). NO inhibitors provide a potential new avenue for treatment of airway diseases (Hesslinger et al. 2009).

Vasoactive intestinal peptide (VIP) is often co-released with NO from NANC nerves. As its name implies, it plays a role in increasing water and electrolyte secretion
into the gut lumen. It also acts as a bronchodilator in the lungs by binding to its receptors VPAC1 and VPAC2. Both receptors are GPCRs coupled to Gαs, which increase cAMP to cause bronchodilation. VIP also has anti-inflammatory effects on mast cells, making it a strong candidate for a therapeutic target. Currently new VIP agonists are being developed for the treatment of COPD and asthma, but there have been some limiting systemic side effects (Mathioudakis et al. 2013).

**Sympathetic Nerves**

While β2 agonists act directly on ASM to cause airway relaxation, there is little evidence that sympathetic nerves play a profound role in modulating airway diameter. β2ARs have low affinity for the primary sympathetic neurotransmitter norepinephrine. Most of their endogenous activity is likely mediated by epinephrine released from the adrenal gland, rather than local regulation by norepinephrine release by nerves. Any sympathetic innervation in the lungs may be directed at the pulmonary vasculature rather than the airways.

**Sensory Nerves**

Small unmyelinated afferent C-fibers transmit signals to and from the lung through the release of sensory neuropeptides. These neuronal messages are also transmitted along the vagus nerve with cell bodies in the nodose and jugular ganglia. A small fraction of sensory nerves also innervates the lung from the dorsal root ganglia located in upper vertebrae of the spine. Calcitonin gene-related peptide (CGRP) is a neuropeptide present in sensory nerves. It is a pain and inflammation signaling protein (Benemei et al. 2009). CGRP binds to the GPCR calcitonin receptor-like receptor (CALCRL) associated with the receptor activity modifying protein 1 (RAMP1) and
signals via activating Gαs. Li et al. (2014) found that mice lacking the RAMP1 protein did not develop allergic sensitization to ovalbumin and produced less IL-4 in the lungs. Recently monoclonal antibody drugs have been created that target CGRP and its receptor have been approved to treat migraine.

Substance P is another major inflammatory neuropeptide released by sensory nerves. It primarily exerts its effects through the GPCR neurokinin type-1 receptor (NK1). NK1 receptors are present on nerves as well as monocytes, macrophages, and epithelial cells (Garcia-Recio & Gascón, 2015). Activation of NK1 receptors on nerves can lead to enhanced release of ACh in response to EFS (Tournoy et al. 2003). Administration of exogenous substance P to mouse trachea causes relaxation. Substance P causes relaxation via release of prostaglandin E₂ and activation of the prostaglandin E₂ type-2 (EP₂) receptor (Fortner, Breyer, and Paul 2001).

**The Nervous System and Asthma**

Asthma is primarily an inflammatory disease. However, there is considerable evidence that the immune system and the peripheral nervous system interact in the lungs (McGovern and Mazzone 2014). In allergic airways, allergen specific IgE antibodies are bound to the surface of mast cells. Antigen binding causes bridging between antibodies, leading to mast cell degranulation. In humans, one of the primary mediators released from mast cells is histamine. Another component released from mast cells after degranulation is serotonin (5-HT). Weigand et al. (2009) demonstrated that, in mice, 5-HT acts on parasympathetic nerves to cause release of ACh. This is the primary driver of allergic airway constriction. In their system, allergic airway constriction was blocked ~100% by the 5-HT receptor antagonist ketanserin and ~75% by atropine. Additionally,
they found mast cells were located within the ASM, proximal to nerve endings. This supports the idea of a natural link between the nerves in the airway and the immune system.

While 5-HT can cause release of ACh, ACh itself can act to cause inflammation, leading to further migration of immune cells into the lungs. In fact, ACh may be pro-inflammatory via muscarinic receptors while also acting as an anti-inflammatory signal via nicotinic receptors. By blocking muscarinic receptors, tiotropium can block the infiltration of eosinophils into both large and small airways (Meurs et al. 2013). Eosinophils and the pulmonary epithelium can release the pro-inflammatory molecule nerve growth factor (NGF), leading to both increased inflammation and nerve remodeling. Bachar et al. (2004) found increased nerve density after chronic treatment with NGF in the mouse superior trachea. They also found that the superior trachea has increased EFS response after 4 days of incubation with nerve growth factor while there was no difference in the inferior trachea. There were no acute differences in fresh culture. Currently anti-NGF monoclonal antibodies are in clinical trials for pain management (Bannwarth and Kostine, 2017).

One prominent class of inflammatory autacoids that are released by the epithelium and white blood cells in the lung are the prostanoids. Prostanoids are a subclass of the arachidonic acid-derived eicosanoids and consist of the prostaglandins, prostacyclins, and thromboxanes. They are formed by cyclooxygenase (COX) -catalyzed conversion of arachidonic acid to PGH₂. From there PGE synthase or PGD synthase can convert PGH₂ into the pro-inflammatory prostaglandins prostaglandin E₂ (PGE₂) and prostaglandin D₂ (PGD₂), respectively. While PGD₂ causes direct bronchoconstriction,
PGE₂ causes bronchodilation. Fevipiprant is a PGD₂ type-2 receptor antagonist currently in Phase III trials for the treatment of asthma (White, Wright, and Brightling 2018). While PGE₂ causes smooth muscle relaxation in humans (via EP₄), it also stimulates nerves (via EP₃) causing coughing, limiting its utility in treating asthma (Maher, Birrell, and Belvisi 2009). More selective agonists may have some benefit in asthma treatment.

Thromboxane synthase converts PGH₂ into the vaso- and broncho-constrictor thromboxane A₂ (TXA₂). Prostacyclin synthase forms PGI₂ (prostacyclin) which induces vaso- and broncho-dilation (Rolin, Masereel, and Dogné 2006). This mix of constrictors and relaxers creates a complex inflammatory environment in the lungs but offers some promising targets for novel therapeutics.

Neuroepithelial bodies (NEBs) are clusters of neuroendocrine cells, a specialized type of epithelial cells. These cell groups are primarily localized near bifurcations in the bronchioles, but not in the lower airways. (Noguchi, Sumiyama, and Morimoto 2015). There they act as chemosensors and are innervated basally by vagal afferent nerves (Chang et al. 2015). They characteristically contain and release the neuropeptide CGRP and may regulate local inflammation in the airways. Because they are innervated, they act as a bridge between the airway epithelium, nervous system, and immune system. Afferent sensory nerve signals (from NEBs or otherwise) transmit inflammatory signals to the brain, which may cause reflex signals to be sent to more centralized immune organs such as the spleen (McGovern and Mazzone 2014). In this way the lung may act as a sensor that regulates organism level inflammation.

At a more clinical level it is difficult to measure the activity of the nervous system in the airways. One method for measuring autonomic nervous system activity is by
measuring heart rate variability (HRV). The frequency of fluctuations between heart beats can be used to measure autonomic activity, with high-frequency (HF) fluctuations corresponding to parasympathetic activity and low-frequency (LF) fluctuations corresponding to a mixture of sympathetic and parasympathetic outflow. The vagus nerve that innervates the heart also innervates the lungs, and the autonomic activity is correlated between the two. Measuring particular frequencies of HRV can be used to study the systemic activity of the autonomic nervous system. While the field of HRV analysis is still young, in a study of 77 children with atopic asthma, Emin et al. (2012) found that asthma severity was correlated with HF (parasympathetic) HRV. This correlation was present even in the absence of asthma stressors. This result is consistent with an overactivation of the parasympathetic nervous system in atopic asthma patients.

**Airways, Nerves, and Inflammation**

Despite decades of study, the role of nerves in asthma remains poorly understood. Only recently has the significance of the interaction between the autonomic nervous system and the immune system become apparent in the lungs. On one hand, ACh plays an anti-inflammatory role in many tissues, including the lungs (Gwilt, Donnelly, and Rogers 2007). On the other hand, cholinergic stimulation of airways causes direct smooth muscle constriction. In fact, severing the vagus nerve in mice has been shown to reverse allergen-induced airway hyper-responsiveness (McAlexander et al. 2015). Nerves are an attractive target for the treatment of asthma. While a variety of new monoclonal antibodies have reached the market to treat eosinophilic asthma (Groot, Brinke, and Bel 2015), there are few treatments for non-eosinophilic severe asthma (Thomson 2016). This
subpopulation may show a unique sensitivity to treatments that target nerves for the relief of asthma symptoms.

One difficulty in studying this system is the complexity of the interactions between the airways, the nervous system, and the immune system. The immune and nervous systems are difficult to study in vitro. Cell-culture is only capable of incorporating limited cell types at one time, while in vivo lungs contain parasympathetic and sensory nerves, eosinophils, ASM cells, epithelium, NEBs, neutrophils, mast cells, and more. Isolated whole tissue experiments incorporate all the native cell types to allow study of their functions on modulating airway tone. Furthermore, it may be necessary to perform wet-lab experiments that have limited cell diversity and use computational modeling to recapitulate nervous or immune system information into the results to produce translatable interpretations of the data.

Objectives

The purpose of these studies was to enhance the understanding of the functional role of the neurotransmitter systems present in the lungs. Mouse lungs were used as a model system for lung innervation because mice are commonly used as model animals for asthma and are available with a variety of genetic knock-outs. To characterize the role of neurotransmitters in modulating constriction and relaxation of the airways, I stimulated isolated mouse airway nerves using electrical field stimulation (EFS). EFS can be used to study the functional innervation of the airways by causing release of neurotransmitters that can be pharmacologically blocked to decipher their roles in airway modulation. My hypothesis was that a computational model can be used in conjunction with experimental results to describe the roles of nerves in the response to EFS in mouse airways.
In the studies in Chapter 2, I develop computational models of neuronal control of airway diameter based on preliminary EFS experiments. Data from inferior trachea EFS experiments were used to design a compartment-based distribution model. Because ACh from parasympathetic nerves is the dominant neurotransmitter in the lung, I initially developed an ACh model with first-order kinetics (the First-Order model) to fit the control data responses; however, this model did not reproduce the multi-phasic nature of the control responses to EFS. To improve the fit of the model to the trachea constriction experiments, a second model that incorporated inhibition of the regulators of parasympathetic neurotransmission (presynaptic M₂ receptors and AChE) at high concentrations of ACh was developed (the Inhibition model). However, the Inhibition model did not dramatically improve the fit of the control data. Capsaicin and indomethacin were added to the tissue bath experiments to remove the influences of sensory nerves and prostanoids, respectively. Pre-treatment with these two drugs produced a response consistent with parasympathetic constriction via a single neurotransmitter, ACh. After experimentally isolating the ACh response, both the First-Order and Inhibition models were able to more accurately predict experimental results, with the Inhibition model producing the lowest sum of squares error across all frequencies. Successfully modeling the ACh component of the EFS response was the first step in establishing a comprehensive model of nerves in the airways.

The studies in Chapter 3 I examine the functional components of the EFS-induced relaxation phase that was eliminated by capsaicin and indomethacin pre-treatment in the studies in Chapter 2. Isolated tracheas from wild-type mice were placed in tissue baths and exposed to EFS and various pharmacological agents. The relaxation kinetics differed
between the inferior (lower) portion of the trachea and the superior (upper) portion of the trachea. PGE$_2$ was found to be a prominent contributor to relaxation in the inferior portion of the trachea. In EFS experiments in precision cut lung slices, the frequency-responses of lower airways were different from those seen in either part of the trachea. Furthermore, maximum constriction appeared to be heterogenous in different lung lobes. Taken together these data support differential distribution of nerve populations and neurotransmitters systems throughout the upper airways and lungs, complicating efforts to model the system.

In Chapter 4 the computational model from Chapter 2 is extended using the additional experimental information from Chapter 3. The results in Chapter 2 established the model foundation, from which additional models could be constructed. Instead of focusing solely on the results from the inferior trachea, a similar modeling procedure was applied to the superior trachea. As with the inferior trachea, the Inhibition model produced the best fit of the superior trachea treated with capsaicin and indomethacin. Finally, I extended the inferior trachea model to include multiple neurotransmitters. Given the results in Chapter 3, PGE$_2$ is a likely candidate for causing the EFS induced relaxation phase. Using this information, PGE$_2$ was added into the model. The Two-Drug model was able to reproduce multi-phasic EFS constrictions in the lower trachea, consistent with experimental observations. Overall, the developed computational model described the roles of nerves in response to EFS. This information will inform future studies into the roles that nerves play in the heterogeneous pathologies of asthma.
CHAPTER 2: DEVELOPMENT OF A COMPUTATIONAL MODEL TO SIMULATE NERVE-MEDIATED CONstriction OF MOUSE TRACHEA

Introduction

Autonomic and sensory nerves containing a variety of neurotransmitters innervate the lungs and control airway diameter. Constriction of mammalian airways is primarily driven by the release of acetylcholine (ACh) from parasympathetic nerves. These cholinergic responses are centrally mediated via parasympathetic fibers in the vagus nerve. ACh released from parasympathetic nerves activates muscarinic type-3 (M₃) receptors on airway smooth muscle (ASM) to cause bronchoconstriction. Electric field stimulation (EFS) of isolated trachea causes nerve depolarization and release of ACh and other neurotransmitters that in turn diffuse across the neuroeffector junction and act on tracheal smooth muscle to cause contraction. Measuring tracheal constriction provides a model system to develop computational methods to simulate constriction of airways.

Drug distribution models build outward from first principles using computational methods to describe the movement of drugs throughout an organism. These models create a rigorous mathematical framework to allow for computer simulation of in vivo processes. While these models are classically used to model drug movement throughout an entire organism, each parameter has a corresponding physical interpretation. In applying this approach to EFS-induced constriction of mouse trachea each electrical pulse becomes the equivalent of a dose of neurotransmitter (i.e. ACh) released into the neuroeffector junction (Figure 1). After release, ACh diffuses across the neuroeffector junction (absorption), where it causes an effect (constriction), while at the same time being broken down by acetylcholinesterase (elimination). Modeling the effects of neuronally released ACh constriction of mouse trachea is a critical first step in creating a
comprehensive model to understand the role of nerves in regulating airway constriction. A complete model may provide a high-throughput screening tool to predict the effects of novel agents on nerve-mediated airway function. Coupled with in vitro and in vivo experiments, this model may also be used to elucidate mechanisms and provide insights into the rational use of drugs to target airway nerves in asthma.
Figure 1 - Physiological Relationship Between a Neuroeffector Junction and a Compartment-Based Model
Methods

Animal experiments were approved by Creighton University’s Institutional Animal Care and Use Committee and were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Tissue Preparation and Electric Field Stimulation

Male CF-1 mice over 6 weeks in age were sacrificed with CO$_2$. The trachea, lungs and heart were removed *en bloc*. The trachea, from the larynx to the bronchial bifurcation, was isolated, cleaned of connective tissue and cut into two segments. The inferior segment, closest to the bronchial bifurcation, was mounted on a custom 3D-printed post (Appendix D) by inserting a metal pin through the tracheal lumen. The trachea was supported on the post by a wire connected to a Grass FT.03 force transducer used to measure contraction. The trachea with support was placed in a glass muscle chamber containing Krebs-Henseleit solution bubbled with 95% O$_2$ and 5% CO$_2$.

Tracheas were equilibrated for at least 1 hour and then stimulated with 60 mM KCl followed by a 20-minute washout. This was repeated 3 times. The custom post allowed two 1-mm thick platinum wire electrodes to be lowered within 4 mm of either side of the trachea. For EFS, tracheas were stimulated for 1 min using a Grass S48 stimulator, followed by a 2-minute rest period. Tracheas were stimulated using parameters previously shown to selectively activate nerve depolarization without directly stimulating muscle contraction (80 V, 1.0 ms pulse duration; Kesler and Canning 1999). Frequencies were varied from 0.1 to 10 Hz at half-log$_{10}$ intervals. Increases in the frequency of stimulation caused proportional increases in nerve-mediated tracheal constriction. Constriction was amplified using a Grass 79E Polygraph, the signal was
digitized at 50 samples/sec using a Data Trac A-D converter and recordings were analyzed using LabScribe software (iWorx Systems Inc. Dover, NH).

First-Order Model

The goal was to model constriction responses of trachea to EFS at all frequencies with a single set of parameters. The open-source R package RxODE was used to model changes in trachea tone in response to EFS. The RxODE package solves systems of differential equations given initial parameters and a drug administration regimen (Wang, Hallow, and James 2016). The time points of administered drug corresponded to the frequency of EFS in this model. At each EFS stimulation time point, a dose of the released neurotransmitter ACh was instantly deposited into the depot compartment ($C_D$), which serves as an ACh reservoir (Figure 1). From the depot compartment, ACh was absorbed into the central compartment ($C_1$).

While in the central compartment ACh can act on $M_3$ receptors to produce a constriction effect. The constriction of ASM in response to ACh is governed by a log dose-response curve. The effective concentration of ACh causing a half maximal constriction effect ($EC50_{ACH}$) was 4.14 µM (Appendix B, Figure 21), a value consistent with published values (Cheng et al. 2002). Constriction effects were normalized to a maximal effect ($E_{max}$) of 1.0 using Equation 1 (Kenakin 1997):

$$Effect = \frac{E_{max} \times C_1}{EC50_{ACH} + C_1}$$ (1)
The First-Order model used first-order rate constants to describe the kinetics of ACh movement in to and out of the central compartment and was composed of a system of two differential equations. The first equation, Equation 2, defined the ACh concentration of $C_D$ while the second, Equation 3, defined the ACh concentration of $C_1$. Absorption into $C_1$ was governed by the first-order rate constant $k_a$. ACh was removed from $C_1$ by first-order elimination processes regulated by the rate constant $k_e$. Best fit values for $k_a$, $k_e$, and the log dose of ACh released from the nerves per pulse (pDose) were found at all frequencies.

\[
\frac{dC_D}{dt} = -k_a C_D \tag{2}
\]

\[
\frac{dC_1}{dt} = k_a C_D - k_e C_1 \tag{3}
\]

**Inhibition Model**

The Inhibition model was developed to include various processes that could modulate the effect of released ACh to cause constriction. Like the First-Order model, the Inhibition model consisted of two differential equations describing the two compartments $C_D$ and $C_1$. Additionally, this model incorporated two new components, with two new unknown parameters for each component, for a total of four additional unknown parameters. Functional forms for the additional components were taken from Macheras and Iliadis (2016). One component modeled inhibition of ACh release, which is analogous to activation of pre-junctional, auto-inhibitory $M_2$ receptors (Equation 4). The second component modeled inhibition of ACh elimination due to saturation of
acetylcholinesterase (AChE)-mediated breakdown at higher concentrations of ACh (Equation 5).

\[
\frac{dC_D}{dt} = -k_a C_D \left(1 - \frac{M_{2_{\text{max}}} \times C_D}{IC_{50_{M2}} + C_D}\right)
\]  

\[
\frac{dC_1}{dt} = k_a C_D \left(1 - \frac{M_{2_{\text{max}}} \times C_D}{IC_{50_{M2}} + C_D}\right) - k_e C_1 \left(1 - \frac{AChE_{\text{max}} \times C_1}{IC_{50_{AChE}} + C_1}\right)
\]

In order to find the best-fit parameters for the Inhibition model, trachea constrictions from the lowest frequency (0.1 Hz) were first used to calculate the \(k_a\), \(k_e\), and pDose using the First-Order model. At this frequency the pulses were temporally distinct from one another and the saturation of AChE and auto-inhibition of ACh release by \(M_2\) receptor activation were assumed to play a negligible role. The parameters \(k_a\), \(k_e\), and pDose were held constant at higher frequencies to allow best fit of the four additional Inhibition model parameters. Auto-inhibition of ACh release by activation of pre-junctional \(M_2\) receptors was governed by the parameters \(M_{2_{\text{max}}}\) and \(IC_{50_{M2}}\). Similarly, the parameters \(AChE_{\text{max}}\) and \(IC_{50_{AChE}}\) were used to describe saturation of AChE at higher ACh concentrations.

**Method of Least Sum of Squares**

To find the best fit to tracheal constriction for each model, an iterative random-walk parameter search was used to establish the set of parameters that minimized the sum of squared differences between the models and experimentally determined tracheal constriction (Kenakin 1997). Briefly, a vector \(P\) of physiologically reasonable initial parameters for each of the unknown parameters was initialized: \(P = [k_a, k_e, \text{pDose}]\).
A new $p_1^*$ was then sampled from a normal distribution with $p_1$ as the mean and a standard deviation of $\lambda \times p_1$. The hyperparameter $\lambda$ provided good convergence of the unknown parameters at a value of 0.1. Using this updated parameter ($p_1^*$), the model was solved, and the sum of squared differences was calculated again. If the sum of squared differences decreased, then $p_1^*$ was accepted and became the new $p_1$, otherwise $p_1^*$ was rejected. The model then chose a new $p_2^*$, followed by a new $p_3^*$ etc. This was repeated 500 times to converge on a final set of parameters. The model parameters were reinitialized, and the process was repeated until 100 sets of parameters were produced for each tissue at each frequency. More details on the method can be found in Appendix A.

**Capsaicin and Indomethacin Treatment**

To block non-ACh neurotransmitter modulation of EFS-mediated tracheal constriction, tracheas were incubated for two hours with 100 µM capsaicin and 10 µM indomethacin. Following incubation with capsaicin and indomethacin tracheas were washed for one hour before a frequency response curve was generated. Capsaicin causes depolarization of sensory nerves and depletion of sensory neurotransmitters while indomethacin blocks cyclooxygenase (COX) and prevents the formation of prostanoids. These treatments are known to regulate EFS-mediated tracheal constriction (Kocmalova et al. 2017 and Waugh et al. 1999). Together, these pharmacological treatments reduced the number of chemical mediators produced during EFS and allowed us to fit the models to constriction produced in a less complex system with fewer unknown parameters.

**Data Analysis and Statistics**

Modeling, parameter fitting, plotting, and statistical analyses were performed with R (3.5.0; R Core Team 2017) using the RStudio graphical user interface (1.1.447;
RStudio Team 2016). Statistical comparisons were made using unpaired two-tailed t-tests with a p-value < 0.05 considered as a significant difference between groups. Data are presented as means ± standard errors. All R code used for fitting the models to tracheal constriction and for analysis can be found at https://github.com/CamKieff/NervePKPDmodel.

**Results**

**Control Trachea EFS Constriction**

Increasing the frequency of EFS increased the Area Under the Curve (AUC) of the constriction of mouse trachea (Figure 2A). The constriction to EFS was multi-phasic and consisted of both contraction and relaxation phases. EFS constriction was blocked by the voltage-gated sodium channel blocker tetrodotoxin (1 µM) and the non-selective muscarinic receptor antagonist atropine (1 nM) (Chapter 3, Figure 7). Taken together, these data indicate that EFS released ACh from nerves that in turn stimulated muscarinic receptors to cause tracheal constriction.
Figure 2 - Control EFS Responses Are Fit by Method of Least Sum of Squares

(A) Representative tracings of tracheas exposed to EFS for untreated control tissue. Tracheas were stimulated for one minute followed by a two-minute relaxation. Tracing has mg tension on the y-axis and time on the x-axis. Stimulation frequencies are indicated along the x-axis. (B) Example fit of the First-Order model at 0.1 Hz. Grey is a 0.1 Hz tracing for Tissue 1. The violet curve is the naive initial parameters for the model. The blue curve is the best fit for the First-Order model after applying the method of least squares.
Control Tissue First-Order Model Fits

The First-Order model was first fit to control tracheal constriction recordings. Using the method of least sum of squares to find best-fit parameters substantially improved the First-Order model fit compared to the initial parameters (Figure 2B). The mean $k_a$ at 0.1 Hz was $2.71 \pm 0.11 \text{ sec}^{-1}$ while the mean $k_e$ was $1.15 \pm 0.10 \text{ sec}^{-1}$. These correspond to half-lives of 0.26 sec and 0.60 sec, respectively. The mean 0.1 Hz pDose was in the micromolar range at $6.06 \pm 0.06$. The best-fit $k_e$ values were consistent across frequencies, with no significant differences in $k_e$ values (Table 1A). Best-fit $k_a$ values were more variable but, with one exception, there were no significant differences in $k_a$ across frequencies (Table 1A). In contrast, the best-fit pDose values varied significantly across frequencies and trended upwards as the stimulation frequency increased, with significant differences in pDose across frequencies (Table 1A). Each of the 100 sets of final best-fit parameters for $k_a$, $k_e$, and pDose were approximately normally distributed. The mean best-fit parameters for the First-Order model across all frequencies were $k_a = 2.32 \pm 0.54 \text{ sec}^{-1}$, $k_e = 1.09 \pm 0.06 \text{ sec}^{-1}$, and pDose = $6.24 \pm 0.06$.

The model assumes that as the EFS frequency increases, only the rate of neurotransmitter release into the neuroeffector junction is changed. If this assumption is correct, a single set of parameters should be able to accurately predict single-tissue responses across all frequencies. Tissue-specific best-fit frequency-spanning parameters were calculated and used in First-Order model simulations (Figure 3, Blue). As a global measure of goodness of fit, the AUC determined from the First-Order model simulations was compared to the experimental constriction recordings. While these sets of frequency-spanning parameters reasonably modeled constriction there were significant
differences in AUC between the First-Order model and constriction recordings. The model predicted a significantly lower AUC at 0.1 and 0.3 Hz with a significantly increased AUC at 10 Hz (p < 0.01) compared to the constriction recordings (Table 2).

**Control Tissue Inhibition Model Fits**

Since the only significant differences across frequencies in First-Order best-fit values were in pDose (Table 1A), frequency-related changes in the dose of ACh were predicted to be responsible for the inability to effectively model tracheal constriction. Therefore, the Inhibition model was developed to assess changes in dose of ACh across frequencies during EFS. The Inhibition model included factors to modulate the rate constants at the different concentrations of ACh present at low versus high frequencies. In this model the $k_a$, $k_e$ and pDose were not individually fit for each frequency. Instead, the best fits for these three parameters were determined at 0.1 Hz, where constrictions were temporally distinct, and then applied across frequencies for each tissue individually.

The Inhibition model assumed that $k_a$, $k_e$, and pDose remained the same across frequencies and that any deviations from this assumption would be captured in the four additional parameters.

The Inhibition model included two components that can change the pDose. One component was pre-junctional inhibition of ACh release by activation of M$_2$ receptors ($M_{2\text{max}}, \text{pIC}50_{M2}$) and the other was saturation of AChE ($AChE_{\text{max}}, \text{pIC}50_{AChE}$). The method of least sum of squares was used to find the best-fit parameters of $M_{2\text{max}}, AChE_{\text{max}}, \text{pIC}50_{M2},$ and $\text{pIC}50_{AChE}$. In contrast to the frequency-dependent parameters in the First-Order model, three of the dose-related parameters in the Inhibition model ($M_{2\text{max}}, \text{pIC}50_{M2},$ and $\text{pIC}50_{AChE}$) did not vary by frequency (Table 1B), and $AChE_{\text{max}}$
only varied at 0.3 Hz compared to 1 and 3 Hz. The mean best fit parameters across all
tissues and frequencies were $M_{2\text{max}} = 0.91 \pm 0.06$, $AChE_{\text{max}} = 0.30 \pm 0.04$, $pIC50_{M2} = 6.13 \pm 0.44$, and $pIC50_{AChE} = 4.43 \pm 0.32$.

As with the First-Order model a single set of Inhibition model parameters should
be predictive across all frequencies. Therefore, tissue-specific best-fit frequency-
spanning parameters were calculated and used in Inhibition model simulations.
Differences in AUC similar to those found using the First-Order model remained after
comparing the AUC from the Inhibition model simulations with the trachea constriction
recordings. In this case, the simulation predicted AUC was significantly lower at 0.1 Hz
and significantly higher at 3 and 10 Hz ($p < 0.01$) compared to constriction recordings
(Table 2). An example of tracheal constriction recordings compared to the Inhibition
model and the First-Order model is shown in Figure 3 (Red).
### Table 1 - Control Tissue Best-Fit Parameter Results

#### A

<table>
<thead>
<tr>
<th>Hz</th>
<th>k&lt;sub&gt;a&lt;/sub&gt; ±</th>
<th>k&lt;sub&gt;e&lt;/sub&gt; ±</th>
<th>pDose ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2.71 ± 0.11</td>
<td>1.15 ± 0.10</td>
<td>6.06 ± 0.06</td>
</tr>
<tr>
<td>0.3</td>
<td>0.24 ± 0.03</td>
<td>1.01 ± 0.09</td>
<td>6.03 ± 0.02</td>
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<tr>
<td>1</td>
<td>1.86 ± 1.00</td>
<td>1.10 ± 0.14</td>
<td>6.08 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>5.37 ± 1.70&lt;sup&gt;&amp;&lt;/sup&gt;</td>
<td>1.11 ± 0.01</td>
<td>6.47 ± 0.11&lt;sup&gt;*&amp;&lt;/sup&gt;&lt;sup&gt;&amp;&lt;/sup&gt;&lt;sup&gt;&amp;&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>1.43 ± 0.55</td>
<td>1.09 ± 0.03</td>
<td>6.57 ± 0.09&lt;sup&gt;*&amp;&lt;/sup&gt;&lt;sup&gt;&amp;&lt;/sup&gt;</td>
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</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th>Hz</th>
<th>M&lt;sub&gt;2max&lt;/sub&gt; ±</th>
<th>AChE&lt;sub&gt;max&lt;/sub&gt; ±</th>
<th>pIC&lt;sub&gt;50M2&lt;/sub&gt; ±</th>
<th>pIC&lt;sub&gt;50AChE&lt;/sub&gt; ±</th>
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<tbody>
<tr>
<td>0.3</td>
<td>0.99 ± 0.01</td>
<td>0.56 ± 0.03</td>
<td>7.03 ± 0.06</td>
<td>6.29 ± 0.20</td>
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<td>1</td>
<td>0.66 ± 0.22</td>
<td>0.20 ± 0.12&lt;sup&gt;&amp;&lt;/sup&gt;</td>
<td>5.00 ± 1.67</td>
<td>2.89 ± 1.68</td>
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<tr>
<td>3</td>
<td>1.00 ± 0.00</td>
<td>0.19 ± 0.06&lt;sup&gt;&amp;&lt;/sup&gt;</td>
<td>6.43 ± 0.11</td>
<td>4.99 ± 0.23</td>
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<td>10</td>
<td>0.99 ± 0.001</td>
<td>0.26 ± 0.10</td>
<td>6.05 ± 0.08</td>
<td>3.56 ± 1.19</td>
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</tbody>
</table>

Results from the (A) First-Order and (B) Inhibition models in the control inferior trachea.

Data are means ± SEMs (n = 4). The asterisk (*) indicates a statistically significant difference (p-value < 0.05) from 0.1 Hz parameter using a one-way ANOVA with a Tukey post-test. A significant difference from 0.3 Hz, &; significant difference from 1 Hz, #; significant different from 3 Hz, $. 
Figure 3 - Example Model Fits to Control Tissue Data

Representative plot of control model results for inferior tissue 1. Each panel represents a different frequency from 0.1 to 10 Hz. The three datasets plotted in each panel are the normalized experimental data tracing (grey), the First-Order model fit (blue), and the Inhibition model fit (red). Each response is normalized to the maximum tissue EFS response (30 Hz) in the presence of capsaicin and indomethacin.
Table 2 - Model Area Under the Curve Results

<table>
<thead>
<tr>
<th></th>
<th>Capsaicin</th>
<th>0.1 Hz</th>
<th>0.3 Hz</th>
<th>1 Hz</th>
<th>3 Hz</th>
<th>10 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>-</td>
<td>1.51 ± 0.07</td>
<td>3.76 ± 0.39</td>
<td>9.69 ± 1.75</td>
<td>11.14 ± 2.27</td>
<td>21.76 ± 2.79</td>
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<tr>
<td>Raw</td>
<td>+</td>
<td>1.84 ± 0.30</td>
<td>6.37 ± 0.99</td>
<td>19.64 ± 3.94</td>
<td>28.20 ± 3.96</td>
<td>47.25 ± 2.78</td>
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<tr>
<td>First-Order</td>
<td>-</td>
<td>0.76 ± 0.14*</td>
<td>2.25 ± 0.39*</td>
<td>6.83 ± 1.04</td>
<td>16.43 ± 1.96</td>
<td>32.92 ± 2.25*</td>
</tr>
<tr>
<td>First-Order</td>
<td>+</td>
<td>2.57 ± 0.52</td>
<td>7.14 ± 1.38</td>
<td>18.00 ± 2.89</td>
<td>32.75 ± 3.62</td>
<td>47.14 ± 2.57</td>
</tr>
<tr>
<td>Inhibition</td>
<td>-</td>
<td>1.06 ± 0.10*</td>
<td>3.15 ± 0.28</td>
<td>9.13 ± 0.64</td>
<td>19.58 ± 1.66*</td>
<td>35.90 ± 2.83*</td>
</tr>
<tr>
<td>Inhibition</td>
<td>+</td>
<td>2.85 ± 0.69</td>
<td>8.62 ± 2.17</td>
<td>19.77 ± 3.97</td>
<td>32.12 ± 3.99</td>
<td>45.78 ± 1.92</td>
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</table>

Table showing AUC results for trachea constriction raw data, First-Order model and Inhibition model best fits. The asterisk (*) indicates a difference between model fit and corresponding raw data. (p < 0.05; two-tailed unpaired t-test). For the Treated tissue data there are no statistically significant differences between either the First-Order or Inhibition model and the raw data.
Tissue Treatment to Isolate ACh Constriction

The contraction to EFS was multi-phasic and consisted of contraction followed by relaxation. The relaxation phase of the EFS response was eliminated in order to study the kinetics of ACh-induced contraction alone by pre-treating the tracheas with capsaicin and indomethacin. As shown in Figure 4, after this treatment, the EFS response consisted of only a single constriction phase. Under this condition, constriction was likely only mediated by ACh, which allowed us to more accurately evaluate factors that can modify the administered dose of ACh in the First-Order and Inhibition models.

![Figure 4 - EFS Responses After Pre-Treatment with Capsaicin and Indomethacin](image)

Representative tracing of tracheas exposed to EFS for tissue after pre-treatment for two hours with 100 µM capsaicin and 10 µM indomethacin followed by a one-hour washout period. Each tissue was stimulated for one minute followed by a two-minute relaxation. Tracing has mg tension on the y-axis and time on the x-axis. Stimulation frequencies are indicated along the x-axis.

Treated Tissue First-Order Model Fits

The experimental trachea tissue recordings after pre-treatment with capsaicin and indomethacin (hereafter referred to as the “Treated” data) were fit using the First-Order
model. As before, the method of least sum of squares estimated best-fit parameters. The mean \( k_a \) at 0.1 Hz was \( 2.83 \pm 0.22 \text{ sec}^{-1} \), while the mean \( k_e \) was \( 1.09 \pm 0.08 \text{ sec}^{-1} \) which correspond to half-lives of 0.25 sec and 0.64 sec, respectively. The mean 0.1-Hz pDose was \( 5.91 \pm 0.07 \). These values were not statistically different from \( k_a, k_e, \) and pDose determined from First-Order model fits to control EFS constriction. Best-fit \( k_e \) and pDose values were not significantly different across all frequencies (Table 3A). With the exception of 0.1 Hz, \( k_a \) values at all other frequencies were also not significantly different (Table 3A). The mean Treated tissue best-fit parameters for the First-Order model across all frequencies were: \( k_a = 0.69 \pm 0.02 \text{ sec}^{-1} \), \( k_e = 0.83 \pm 0.09 \text{ sec}^{-1} \), and pDose = \( 5.84 \pm 0.08 \).

In addition to least sum of squares, a second method was also used to independently determine rate constants for the trachea constrictions. The \( k_a \) and \( k_e \) were systematically derived using the curve stripping method described in Welling (1986). The 0.1-Hz curves were used because the constrictions were temporally distinct, and several values could be found per tissue. Treated tissues were used because the 0.1 Hz constriction maxima did not vary from pulse to pulse (Appendix B, Figure 20). The mean curve-stripping \( k_e \) was \( 1.06 \pm 0.07 \text{ sec}^{-1} \), which was not significantly different from the least sum of squares \( k_e \) of \( 1.09 \pm 0.08 \text{ sec}^{-1} \). Adding a second elimination phase to the curve stripping procedure did not significantly improve the fits to the experimental data (data not shown). The curve-stripping \( k_a \) value of \( 2.48 \pm 0.17 \text{ sec}^{-1} \) was also not different from the least sum of squares \( k_a \) value of \( 2.83 \pm 0.22 \text{ sec}^{-1} \). Comparing the curve stripping values to the method of least squares determined values supports the use of this method for finding best-fit parameters.
Tissue-specific frequency-spanning parameters were calculated for Treated tissue constriction recordings. These parameters accurately modeled constriction recordings using the First-Order model. An example of Treated tracheal constriction recordings compared to the First-Order model fit is shown in Figure 5 (Blue). There were no significant differences in AUC, a measure of goodness of fit, between the First-Order model and Treated tissue constriction recordings across all frequencies (Table 2). Thus, when only the contractile neurotransmitter ACh is present the First-Order model more accurately predicts the EFS constrictor response.

**Treated Tissue Inhibition Model Fits**

To determine whether the frequency dependence observed in the best fit $k_a$ parameter in the Treated tissue constriction First-Order model fits was related to pre-junctional activation of $M_2$ receptors and saturation of AChE, the Inhibition model was applied to the Treated tissue data. In a system where only the contractile neurotransmitter ACh produced constriction, neither $M_{2_{\text{max}}}$, $\text{AChE}_{\text{max}}$, nor $\text{pIC}_{50}\text{AChE}$ varied among frequencies (Table 3B). However, $\text{pIC}_{50}M_2$ varied, with significant differences at 0.3 Hz compared to 3 and 10 Hz and at 1 Hz compared to 10 Hz (Table 3B). The mean best fit parameters across all frequencies were $M_{2_{\text{max}}} = 0.98 \pm 0.002$, $\text{AChE}_{\text{max}} = 0.62 \pm 0.13$, $\text{pIC}_{50}M_2 = 6.52 \pm 0.10$, and $\text{pIC}_{50}\text{AChE} = 5.79 \pm 1.00$.

These best-fit frequency-spanning parameters were then used in Inhibition model simulations of the Treated tissue constriction recordings. As with the First-Order model, this set of frequency-spanning parameters accurately modeled constriction recordings (Figure 5, Red). The AUC values of the Inhibition model fits were not significantly different than the tissue constriction recordings across all frequencies (Table 2).
Although the mean difference between the Inhibition model and the constriction recordings (19.4 ± 5.2) was less than the difference between the First-Order model and the constriction recordings (22.3 ± 5.3), these values were not significantly different. However, consistent with a better fit of the Inhibition model, the total sum of squares for the Inhibition model (388) was less than the First-Order model (447). Thus, when considering processes that alter ACh dose, the Inhibition model produced a slightly better fit to the tissue constriction recordings.
### Table 3 - Treated Tissue Best-Fit Parameter Results

#### A

<table>
<thead>
<tr>
<th>Hz</th>
<th>$k_a$</th>
<th>$k_e$</th>
<th>pDose</th>
</tr>
</thead>
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<tr>
<td>0.1</td>
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<td>1.09 ± 0.08</td>
<td>5.91 ± 0.07</td>
</tr>
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<td>0.3</td>
<td>0.22 ± 0.02*</td>
<td>0.80 ± 0.09</td>
<td>5.89 ± 0.05</td>
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<td>0.84 ± 0.10</td>
<td>5.73 ± 0.12</td>
</tr>
<tr>
<td>3</td>
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<td>0.76 ± 0.12</td>
<td>5.91 ± 0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.11 ± 0.04*</td>
<td>0.67 ± 0.14</td>
<td>5.75 ± 0.14</td>
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</table>

#### B

<table>
<thead>
<tr>
<th>Hz</th>
<th>$M_{2\text{max}}$</th>
<th>$AChE_{\text{max}}$</th>
<th>pIC$_{50M2}$</th>
<th>pIC$_{50AChE}$</th>
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<tr>
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<td>0.64 ± 0.23</td>
<td>5.99 ± 0.08*</td>
<td>3.88 ± 1.29</td>
</tr>
</tbody>
</table>

Results from the (A) First-Order and (B) Inhibition models in the trachea after pretreatment with capsaicin and indomethacin. Data are mean ± SEM (n = 4). The asterisk (*) indicates a statistically significant difference (p-value < 0.05) from 0.1 Hz parameter using a one-way ANOVA with a Tukey post-test. A significant difference from 0.3 Hz, &; significant difference from 1 Hz, #; significant different from 3 Hz, $. 
**Figure 5 - Example Model Fits to Treated Tissue Data**

Representative plot of inferior trachea (tissue 1) response after pre-treatment with capsaicin and indomethacin. Each panel represents a different frequency from 0.1 to 10 Hz. The three datasets plotted in each panel are the normalized experimental data tracing (grey), the First-Order model fit (blue), and the Inhibition model fit (red). Each response is normalized to the maximum tissue EFS response (30 Hz) in the presence of capsaicin and indomethacin.
Discussion

ACh is the primary neurotransmitter released from nerves stimulated by EFS in the lungs. EFS responses were shown to be blocked by the muscarinic receptor antagonist atropine and the neuron-specific sodium channel blocker tetrodotoxin (Chapter 3, Figure 7). A first-order kinetic model with a single neurotransmitter, consistent with the hypothesis that ACh is the only neurotransmitter, was developed and applied to control trachea constriction experimental results. A second model that incorporated inhibition of ACh release by activation of M2 receptors and saturation of AChE metabolism of ACh was developed. This model did decrease the frequency-dependence of the model parameters but did not accurately predict the AUC of the trachea constriction responses.

A model of ACh release was not sufficient to explain the untreated control EFS experimental results. While constriction is primarily driven by neuronally released ACh, the multi-phasic experimental results in the untreated data suggest that multiple neurotransmitters can be released by EFS. Treatment with capsaicin and indomethacin reduced the number of neurotransmitters in the experimental system and isolated the ACh component for modeling. This profoundly increased the fit of both the First-Order model and the Inhibition model. Neither model had a mean AUC statistically different from the Treated trachea constriction data. Both models achieved the project goal: a model that fits all frequencies with a single set of parameters.

Nerves in both the human and the mouse lung release a complex mixture of neurotransmitters from various nerve populations. Despite the elimination of sensory nerve neurotransmitter release and prostaglandin production, inhibitory non-adrenergic,
non-cholinergic nerves are also known to co-release nitric oxide and vasoactive intestinal peptide that are not accounted for in the model (Szema et al. 2006). Neurotransmitters also act on other cell types in the trachea besides airway smooth muscle cells to cause responses. Neurotransmitters exert biological effects on normal epithelium, eosinophils, and even other local nerve cells. Of particular interest in asthma would be the interaction between the nervous system and inflammatory immune responses in the lung. Mast cells in the lung are known to interact with cholinergic and sensory nerves and to induce bronchoconstriction (Cyphert et al. 2009). Furthermore, specialized neuroepithelial bodies that contain and released the neuropeptide CGRP are present in the airways near bifurcations. Since these experiments used the lower portion of the trachea, it is possible that these play a significant role in this tissue and may not have been adequately depleted by capsaicin.

Modeling a single constriction phase is useful for establishing a model structure as a proof of concept compared to modeling a mixture of constriction and relaxation phases. The models presented here have established the ACh-related kinetic parameters of EFS. By using the parameters solved for in this pilot study, it will be much easier to solve for the rate constants and other unknown parameters with more complex experimental conditions. For instance, drugs that block specific sensory neuropeptide or specific prostanoid receptors could be added experimentally to generate kinetic parameters that model these components of airway innervation. Conceptually, additional neurotransmitters could easily be incorporated using modified versions of the same differential equations used in these models. Additional neurotransmitter effects could
then be summed together using either the Bliss Independence Model (Foucquier and Guedj 2015) or direct summation of dose-response curves (Di Veroli et al. 2015).

The computational models make several limiting assumptions. In the First-Order model it is assumed that there is only a single population of relevant receptors, the M₃ receptors, and that their activation leads directly to constriction of the trachea. After activation of the M₃ receptor, there are several processes before ASM contraction. This complicates direct interpretations of \( k_a \), \( k_e \), and the other parameters. They could be ensemble constants of different processes or represent a single unspecified rate-limiting process. However, these processes are likely rapid and may be contained within the respective parameters. This model also assumes that each pulse of EFS releases a constant dose of ACh. This simplification is likely incorrect as there is likely pre-synaptic inhibition when concentrations of ACh reach a certain concentration. These limitations are partially manifested in the first-order absorption and elimination constants. The Inhibition model circumvents these limitations to a degree by allowing for M₂ receptor mediated pre-synaptic inhibition and saturation of AChE. However, the Inhibition model still relies only on the M₃ receptors to cause constriction and does not incorporate any of the intracellular machinery into the model.

The restricted compartmental construction of the models introduces additional limitations. This is both a feature and a bug. The limited compartmental design has well defined mathematics from years of pharmacokinetic modeling of organism-level systems. However, it forces all pre-constriction processes into a single first-order rate constant \( k_a \), and all elimination processes into \( k_e \). Additionally, the central effect compartment (C₁) represents an ensemble of heterogeneous neuroeffector junctions. These junctions can
have different sizes, neuronal density, and/or receptor populations in the native tissue. It may not be reasonable to assume that this collection of junctions acts as a homogeneous whole. Indeed, experiments have shown that different portions of the trachea respond differently to EFS (Chapter 3). This indicates that model parameters would also vary, and future studies will require analysis of the superior airways by the model.

The level of detail of this model is appropriate for the stated goals of drug discovery and evaluation. It is not so abstract as to have no interpretability, yet the constants derived from the model correspond to physical processes. Computational drug evaluation techniques are becoming more popular as they dramatically reduce costs of drug screening. Improving modeling and simulation methods for use in in silico clinical trials is an FDA Center/Office Regulatory Science Research Priority Area (Food and Drug Administration 2018). Especially at low EFS frequencies, this model allows for the analysis of the kinetics of drugs that target nerves in airway diseases. Blockade of the dominant ACh effect with atropine could elucidate the kinetics and responses of other neurotransmitters. These neurotransmitters can then be added back into the model. Furthermore, similar EFS experiments can be performed to study the role of nerves in other innervated tissue types such as the aorta, pulmonary artery, vas deferens, and uterus. Ultimately, this model is a strong start, but more work needs to be done to realize its full potential in pushing forward the study of airway innervation in asthma.
CHAPTER 3: DISTRIBUTIONAL DIFFERENCES IN FUNCTIONAL NEUROTRANSMITTER RELEASE IN MOUSE AIRWAYS

Introduction

As verified in Chapter 2, ACh is the primary driver of contraction in mouse trachea. At the same time, there is a strong relaxation phase that was blocked with capsaicin and indomethacin. To study the causes of this nerve-induced relaxation phase in mouse airways, electric field stimulation (EFS) was used to depolarize nerves innervating isolated airways. Nerve depolarization provokes release of neurotransmitters that diffuse across the neuroeffector junction and act on tracheal smooth muscle. Isolated tracheas were cut into superior and inferior segment and exposed to EFS in organ baths. The acute functional roles of EFS-released neurotransmitters on airway smooth muscle (ASM) contraction and relaxation was studied by measuring trachea tone. Similarly, precision cut lung slices (PLCS) were exposed to EFS to study the neurotransmitter populations and functional roles in lower airways. Instead of measuring tone, images of the EFS-induced constriction of these airways was measured to dissect the neurotransmitter effects. Pharmacological agents were used to block receptors, isolating the different neurotransmitters and their mechanisms, allowing the study of the heterogeneous nerve populations in mouse lungs.

Methods

Experiments using animals were approved by Creighton University’s Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.
Electric Field Stimulation

CF-1 mice over 6 weeks of age were sacrificed with CO₂. The trachea, lungs, and heart were removed en bloc. The trachea, from the larynx to the bronchial bifurcation, was isolated and cleaned of connective tissue. The trachea was cut into two segments between the median tracheal rings. The inferior segment, closest to the bronchial bifurcation and the superior segment, closest to the larynx. The trachea was mounted on a custom 3D-printed post designed with TinkerCAD software (www.tinkercad.com) and printed using a Statasys3D printer from ABS plastic in the Reinert-Alumni Memorial Library at Creighton University (Appendix D).

Figure 30). The tissue was supported on the post by a wire connected to a Grass 79E Data Recording System force transducer in a glass bath filled with Krebs-Henseleit solution (final concentrations in mM: NaCl 118.4, KCl 2.7, CaCl₂ 2.5, MgSO₄ 1.18, KH₂PO₄ 1.18, NaHCO₃ 25, Dextrose 11.1, Na₂Ca EDTA 0.029) and bubbled with 95/5% O₂/CO₂.

The custom post allowed two 1-mm-thick platinum wire electrodes to be lowered within 40 mm of either side of the trachea. Tracheas were equilibrated and pre-stimulated with 60 mM KCl three times prior to the start of EFS experiments. Tracheas were stimulated for 1 minute using the platinum electrodes connected to a Grass S48 stimulator. Stimulation was followed by a 2-minute rest period. The tissues were stimulated using parameters known to selectively stimulate nerve depolarization without directly stimulating muscle contraction (80 V, 1.0 ms pulse duration). Tissues were

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stimulated at 0.1, 0.3, 0.7, 1, 3, 7, 10, 15, and 30 Hz. Increases in the number of pulses per second increased the number of nerve depolarizations, and, subsequently, increased the quantity of neurotransmitter release. Recordings were digitized at 50 samples/second using the LabScribe software (iWorx Systems Inc. Dover, NH).

**Drug Treatments**

After isolation and equilibration, tracheas were exposed to a full EFS frequency-response curve followed by a 30-minute washout period. Each experiment then involved administration of different pharmacological tools (reported trial numbers (n) are in the format n = inferior, superior). The non-selective muscarinic receptor antagonist atropine (1 nM; n = 4, 4) or the voltage-gated sodium channel blocker tetrodotoxin (1 µM; n = 7, 5) were added to the bath for 30 minutes before a second frequency-response curve to EFS.

Other tracheas were incubated for two hours with 100 µM capsaicin and 10 µM indomethacin to eliminate relaxation and isolate the ACh-specific constriction phase (n = 5, 7). Capsaicin activates the TRPV1 ion channel located on sensory nerves. Continued activation of TRPV1 leads to depletion of sensory neurotransmitters. Indomethacin blocks the enzyme cyclooxygenase (COX), therefore inhibiting prostanoid formation. Following treatment, the tracheas were washed for one hour and then re-stimulated for another complete frequency-response curve.

The drugs CGRP$_{(8-37)}$ and CP-99994 were used to isolate the roles of different neuropeptides in EFS. CGRP$_{(8-37)}$ (12.5 µM, n = 5, 5) is a peptide fragment of the calcitonin gene-related peptide (CGRP) that blocks CGRP receptors. CGRP is a neuropeptide present in sensory nerves and neuroepithelial bodies. It plays a role in pain
and inflammation signaling (Benemei et al. 2009). CP-99994 (100 nM, n = 5, 4) is a small molecule antagonist selective for the NK1 receptor. The NK1 receptor is the primary receptor activated by Substance P, a major inflammatory neuropeptide released from sensory nerves. After EFS and a 30-minute washout period, tracheas were treated with drugs for 30 minutes before beginning another EFS frequency-response stimulation.

Exposure to capsaicin alone is toxic to the isolated trachea, but it is possible to give indomethacin (10 µM, n = 5, 4) in the absence of capsaicin. Treatment with indomethacin inhibits the formation of prostanoids by COX. PF-04418948 (100 nM, n = 6, 5) is a prostaglandin E2 (PGE2) type-2 receptor (EP2) antagonist. This receptor is present on tracheal smooth muscle cells and is Gαs-coupled. Activation causes smooth muscle relaxation in mouse trachea (Birrell et al. 2012). L-798,106 (200 nM, n = 5, 4) is a PGE2 type-3 receptor (EP3) antagonist. This receptor is believed to present on nerves and acts to inhibit neurotransmitter release (Maher, Birrell, and Belvisi 2009).

In separate CF-1 mouse tracheas, tracheas were pre-contracted with 1 µM ACh. After the ACh constriction plateaued, increasing doses of the neuropeptide CGRP (1 x 10^{-10} M to 1 x 10^{-7} M, at log_{10} intervals) were added to the trachea to measure relaxation. Similarly, other fresh tracheas were pre-contracted with 10 µM ACh and increasing doses of substance P (1 x 10^{-10} M to 3 x 10^{-6} M at half-log_{10} intervals) were added.

**Precision Cut Lung Slices**

CF-1 mice over 6 weeks of age were sacrificed using CO2 and opened along the ventral midline. Gelatin (6%, 0.4 mL) was injected into the right ventricle to perfuse the pulmonary arteries. The trachea was cannulated and injected with ~1.3 mL of 1.8% agarose followed by a 0.2-mL bolus of air. The mouse was kept at 4 °C for 40 minutes.
before the lung lobes were separated and placed into ice-cold Hank’s balanced salt solution (HBSS). The hardened lungs were sliced on a VT1200 vibratome (Leica Biosystems, Nußloch, Germany) set at 200 μm. Slices were placed into 1.5 mL of Minimum Essential Medium (MEM) overnight in a 12-well plate at 37 °C.

Slices were placed into an RC-27NE2 (Warner Instruments, Hamden, CT) perfusion chamber with two 1-mm platinum electrodes on either side of the chamber and perfused with HBSS warmed to 37 °C (Appendix B, Figure 22). Images were recorded on an inverted microscope using a Moticam 2300 digital camera (Motic, Hong Kong). Slices were stimulated with EFS for 3 min (1.0-ms duration, 50 V, 1 - 30 Hz) to selectively depolarize nerves followed by a 5-minute relaxation period. Images were recorded at 3-second intervals during EFS. Airway cross-sectional area was measured using a custom ImageJ script. After control frequency-response curves had been generated, tissues were treated for 30 minutes with 10 mM MgSO₄ or 10 μM atropine followed by an additional frequency-response curve.

Statistics

Because the responses were multi-phasic, with changes in the rates of contraction, relaxation, and maximum effect, the Area Under the Curve (AUC) was used as the primary experimental outcome in isolated trachea experiments. Frequency-response plots were generated with the Prism software (version 5, GraphPad, La Jolla, CA). Plots were fitted using non-linear regression with the baseline variable fixed at zero. Due to the abnormal shape of the frequency-response curves, the AUC responses at 0.1 and 30 Hz were duplicated and modeled at 0.03 and 100 Hz, respectively, to allow for convergence of the curve fit. Trachea frequency-response comparisons for individual frequencies were
with two-tailed paired t-tests. Curve maximum, logEF50, and Hill Slope were all compared using unpaired two-tailed t-tests. An F-test was used to determine if the treated and un-treated frequency-response curves could be fit with a single set of parameters or if the treatment caused a significant change in curve shape. A p-value < 0.05 was considered to be statistically significant.

Results

Responses to EFS

Response tracings differed between the superior and inferior sections of the trachea. Both superior and inferior tracheas had rapid constriction phases at the initiation of EFS. Superior tracheas reached their maximum contraction rapidly and had a slow relaxation phase, as seen in Figure 6A. Inferior tracheas, on the other hand, reached their maximum contractions more quickly, which were then followed by a rapid relaxation phase (Figure 6B). This difference was conserved across all frequencies. Because both the EFS response maxima and curve shapes were altered by drug treatments, the AUC was used as a holistic measurement of treatment response.
Figure 6 - Superior and Inferior Trachea Tracings

Representative tracing of tracheas exposed to EFS for (A) superior control tissue in red and (B) inferior control tissue in blue. Inferior trachea responses displayed a characteristic rapid relaxation phase. Superior tracheas had a much slower relaxation. Each tissue was stimulated for one minute followed by a two-minute relaxation. Tracing has mg tension on the y-axis and time on the x-axis. Stimulation frequencies are indicated along the x-axis.
**EFS Treatment Is Blocked by Tetrodotoxin and Atropine**

Treatment with 1 µM tetrodotoxin decreased the AUC response to EFS at every frequency in both the superior and inferior tracheas (Figure 7A). This confirms that EFS is activating nerves and stimulating release of neurotransmitters to modulate trachea tone. Though responses at higher frequencies in the superior trachea were reduced, they were not completely blocked. In the superior trachea the mean AUC response at 30 Hz was reduced by 69% after tetrodotoxin treatment, compared to a 91% reduction in the inferior trachea. Atropine (1 nM) also reduced EFS mean responses at each frequency. Similar to tetrodotoxin treatment, the AUC reduction at the highest frequency for the superior trachea was lower (72%) than that for the inferior trachea (87%) at the same frequency (Figure 7B). Both the superior and the inferior trachea EFS responses were blocked by the Ca²⁺ channel blocker MgSO₄ (Appendix B, Figure 23). Taken together these data indicate that the constriction phase of EFS is neuronal in origin and primarily mediated by EFS-induced ACh release.
Figure 7 - Tetrodotoxin and Atropine EFS Responses

Frequency-response curves in the inferior (left) and superior (right) tracheas. (A)

Treatment with the voltage-gated sodium channel blocker tetrodotoxin (1 µM; n = 7, 5) (B) Treatment with the non-selective muscarinic receptor antagonist atropine (1 nM; n = 4, 4). Both treatments effectively blocked the constrictive response to EFS in isolated mouse tracheas. Responses are AUC normalized to maximum KCl constriction. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests)
Isolation of ACh Responses

Capsaicin and indomethacin were used to remove the effects of sensory nerves and prostanoids, respectively. This isolated the parasympathetic nerve responses to EFS, primarily the ACh component. Capsaicin and indomethacin pre-treatment abolishes the relaxation phase in both the inferior trachea (Figure 8) and the superior trachea responses (data not shown). Qualitatively, the EFS response tracings shifted from multi-phasic responses to responses with a single constriction phase. In the inferior trachea this led to a profound increase in the frequency-response curve maximum from 3250 ± 409 to 5690 ± 486 (p = 0.0049; Figure 9A). In contrast, there was no statistically significant change in the maximum for the superior trachea (Table 4). Treatment with capsaicin and indomethacin also produced a statistically significant (p = 0.021) 3.9-fold shift in the half-maximal effective frequency (EF50) of the frequency-response curve (Table 5). There was no significant change in the EF50 for the superior trachea. While no individual parameter was statistically different in the superior trachea, pre-treatment with capsaicin and indomethacin did produce a measurable treatment effect in the frequency-response curve (F = 3.75, p = 0.012). This curve change was modest compared to the change in the inferior trachea (F = 49.7, p < 0.0001).

Both capsaicin and indomethacin can affect the production and release of a variety of molecules. Additional experiments to decipher which components were necessary for causing the relaxation phases in the two segments of trachea were necessary.
Figure 8 - Inferior Trachea Tracings Before and After Pre-Treatment with Capsaicin and Indomethacin

Representative tracing of inferior tracheas exposed to EFS for (A) untreated control tissue and (B) tissue after pre-treatment for 2 hours with 100 μM capsaicin and 10 μM indomethacin. Untreated tissues had a characteristic inferior rapid relaxation phase. After treatment however, no relaxation phase was present. Each tissue was stimulated for one minute followed by a two-minute relaxation. Tracing has mg tension on the y-axis and time on the x-axis. Stimulation frequencies are indicated along the x-axis.
Figure 9 - Capsaicin with Indomethacin and Indomethacin Alone EFS Responses
Frequency-response curves in the inferior (left) and superior (right) tracheas. (A) Pretreatment with 100 µM capsaicin and 10 µM indomethacin (n = 5, 7); (B) Treatment with indomethacin alone (10 µM; n = 5, 4). No changes in the curve parameters were detected in the superior trachea. Responses are AUC normalized to maximum KCl constriction. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests).
Neuropeptide Responses in the Inferior Trachea

Capsaicin pre-treatment depletes all sensory neuropeptides. Substance P is one known sensory neurotransmitter. Substance P acts primarily through the neurokinin type-1 (NK1) receptor. However, treatment with the NK1 receptor antagonist CP-99994 did not alter either the maximum (Table 4) or EF50 (Table 5A and B) in either the superior or inferior trachea (Figure 10A). Best-fit curves between CP-99994-treated and -untreated EFS responses were not statistically different and no individual frequency responses were different. Thus the activation of NK1 receptors by released Substance P does not appear to play a role in the acute relaxation response to EFS.

CGRP is another neuropeptide also released from sensory nerves. Qualitatively, treatment with CGRP\(^{(8-37)}\) reduced the relaxation phase in the inferior trachea responses to EFS. The responses were not as dramatic as treatment with capsaicin and indomethacin. While the inferior trachea best-fit curves (Figure 10B) were significantly different (F = 10.4, p < 0.0001), there was no significant change in either the maximum (p = 0.304, Table 4) or the EF50 (p = 0.161, Table 5A). The superior trachea also had a significant change in the shape of the frequency-response curve (F = 22.2, p < 0.0001). Interestingly, instead of reducing the relaxation phase and increasing the AUC, blockade of CGRP receptors caused a decrease in the frequency-response curve maximum from 6660 ± 410 to 4820 ± 274 (p = 0.006, Table 4). CGRP\(^{(8-37)}\) did not alter the EF50 values in the superior trachea (Table 5B).
Figure 10 - CP-99994 and CGRP(8-37) EFS Responses

Frequency-response curves in the inferior (left) and superior (right) tracheas. (A) Treatment with the NK1 receptor antagonist CP-99994 (100 nM, n = 5, 4); (B) Treatment with the CGRP receptor antagonist CGRP(8-37) (12.5 µM, n = 5, 5). Responses are AUC normalized to maximum KCl constriction. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests).
While treatment with CGRP$_{(8-37)}$ reduced the relaxation phase of the lower trachea at high frequencies, direct administration of CGRP to tracheas pre-constricted with ACh did not produce relaxation at any tested dose (Figure 11A). On the other hand, CP-99994 did not produce any changes in the EFS frequency response curves. However, direct addition of substance P caused relaxation at pre-contracted trachea (Figure 11B). It is possible that there are receptors to substance P in the trachea, but that EFS does not cause its release. The relaxation effect appears to be more significant in the inferior trachea; however, more trials will be needed to achieve statistical significance.

*Figure 11 - Effects of Exogenous CGRP and Substance P*

Dose response relationships after addition of (A) CGRP (n = 4, 3) or (B) Substance P (n = 2, 2). Data for the inferior trachea are shown in blue and the superior trachea in red. Tissues were pre-contracted with ACh ((A) 1 µM, (B) 10 µM). Effects are normalized to the stable relaxation to ACh. Points are means ± SEMs.
Activation of EP\textsubscript{2} Receptors by PGE\textsubscript{2} in the Relaxation Response to EFS

Indomethacin alone almost completely inhibits the relaxation phase of EFS induced constriction. In the inferior trachea, indomethacin shifted the EF50 from 2.25 Hz to 0.85 Hz (Table 5A), though this effect did not achieve statistical significance (p = 0.051). While the EF50, maximum, and Hill slope parameters were not individually significant, taken together the best-fit curves were statistically significantly different (F = 21.4, p < 0.0001, Figure 9B). The fold change in the superior trachea EF50 was less pronounced (1.28) and not significant (p = 0.817, Table 5B). There was no change in curve shape for the superior trachea.

One significant product of COX activity blocked by indomethacin is PGE\textsubscript{2}. There are four PGE\textsubscript{2} receptors (EP\textsubscript{1-4}). The EP\textsubscript{2} receptor is reported to be the primary driver of the ASM relaxation effect of PGE\textsubscript{2} in mouse trachea. In humans these effects are believed to be driven by the EP\textsubscript{4} receptor (Benyahia et al. 2012). Both receptors are G\alpha\textsubscript{s}-coupled and relax the airways by increasing intracellular levels of cAMP. Treatment with the EP\textsubscript{2} receptor antagonist PF-04418984 produced a 3.24-fold shift in the EF50 (Table 5A) in the inferior trachea (p = 0.046). The treated and un-treated frequency-response curves for the inferior trachea were best-fit by different sets of parameters (F = 15.1, p < 0.0001, Figure 12A). PF-04418984 did not produce a statistically significant change in the frequency-response curve maximum value for either the inferior or superior trachea (Table 4). PF-04418984 also failed to alter the EF50 or curve shape for the superior trachea (Table 5B).

The EP\textsubscript{3} receptor is expressed pre-synaptically where it inhibits neurotransmitter release. It plays a role in modulating sensory nerve activity in chronic cough. T with the
EP$_3$ receptor antagonist L-798,106 did not produce any statistically significant effects (Figure 12B). The sets of best-fit parameters needed to fit the frequency-response curves were not statistically different between treated and un-treated EFS responses in either the inferior (F = 1.31, p = 0.274) or the superior (F = 1.04, p = 0.381) trachea.
Figure 12 - PF-04418984 and L-798,106 EFS Responses

Frequency-response curves in the inferior (left) and superior (right) tracheas. (A) Treatment with the EP$_2$ receptor antagonist PF-04418948 (100 nM, n = 6, 5); (B) Treatment with the EP$_3$ antagonist L-798,106 (200 nM, n = 5, 4). Responses are AUC normalized to maximum KCl constriction. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests)
PCLS Results

Unlike stimulation in the trachea, AUC analysis in PCLS did not display any relaxation responses following EFS (data not shown). Instead, PCLS responses exhibited altered maximum percent constriction to EFS. Treatment of PCLS with MgSO₄ to block the influx of calcium into neurons during EFS stimulation prevented depolarization and neurotransmitter release (Figure 13A). Treatment with MgSO₄ did not have any effect on airways constriction to ACh (Figure 13B), suggesting that the effect of MgSO₄ is specific to nerves and does not block constriction of ASM.

![Figure 13 - Neuronal Specific Blockade of EFS by MgSO₄ in PCLS](image)

Frequency response curves showing percent constriction to EFS of airways in PCLS in the absence or presence of 10 mM MgSO₄. EFS responses were significantly (* p < 0.05, n = 3) reduced at frequencies greater than 3 Hz. (B) ACh dose-response curves of airways in PCLS without or with 10 mM MgSO₄. There was no significant difference in percent constriction caused by ACh (n = 3). Responses are percent reduction in baseline airway area. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests)
In contrast to data in tracheal sections, atropine did not cause a significant decrease in the maximum constriction to EFS in PCLS (Figure 14). Furthermore, the effect of atropine was frequency dependent. At low frequencies, 1 and 3 Hz, the response to EFS was reduced by 100% and 65% respectively. However, at frequencies greater than 3 Hz, the constriction responses to EFS were insensitive to atropine. Non-linear regression fits were statistically different (F = 6.55, p = 0.0002).

PCLS slices were taken from all locations throughout the mouse lung. Mouse lungs have 5 separate lung lobes. The left lung is a single large lobe, but the right lung consists of 4 distinct lobes. Two of the lobes that produced the highest number of viable slices were the Left (L) lobe and the Right-Middle (RM) lobe. Airways from the RM lobe constricted more in response to EFS than the responses from airways in the L lobe at 1, 3, and 10 Hz (Figure 15). Maximum responses between the two lobes were not significantly different. The constriction to KCl was not different between the two lobes. This may indicate differential nerve population expression between lobes.
Figure 14 - PCLS Atropine Response

Frequency response curves of airway constriction to EFS in the absence (n = 30 - 49) and presence of 10 μM atropine (n = 9 - 10). Responses are % reduction in baseline airway area. Points are mean ± SEM. * p < 0.05
Figure 15 - PCLS Constriction to EFS in Separate Lung Lobes

Frequency response curves of PCLS airway constriction in slices taken from two different lobes of the mouse lungs. The responses from PCLS from the Left Lobe (L; n = 5) are in blue while the responses of PCLS from the Right-Middle Lobe (RM; n = 9) are in black. Responses are percent reduction in baseline airway area. Points are means ± SEMs. *, p < 0.05

Discussion

The studies in Chapter 2 provided suggestive evidence for distributional differences in neuronal populations throughout the mouse lung, and these were further investigated here. Qualitatively, responses between the inferior and the superior trachea appeared to differ in shape. The shape of the untreated control tracings displayed different relaxation kinetics that occurred at all frequencies. While the superior trachea had a slow relaxation after its peak constriction, the inferior trachea had a rapid relaxation phase that eventually plateaued. Additionally, the responses of lower airways in PCLS
had no distinct relaxation phase in response to EFS. These responses also differed from one lung lobe to another.

Inferior trachea responses were consistently blocked by both atropine and tetrodotoxin at all frequencies. However, the superior trachea was insensitive to both atropine and tetrodotoxin at the highest frequencies. This difference could indicate an increase of nerve density in superior tracheas, which would allow EFS to overcome muscarinic receptor antagonism and sodium channel blockade at the highest frequencies. Alternatively, superior tracheas may be more prone to direct ASM stimulation by EFS. In PCLS, a different neuronal blocker, MgSO₄, was used. MgSO₄ completely blocked EFS responses without directly affecting smooth muscle constriction. Airways in PCLS had a frequency-dependent sensitivity to atropine. Atropine was able to block low frequency EFS-induced constriction, but not at frequencies above 3 Hz. Because of the complete blockade by MgSO₄, direct ASM stimulation is not a likely explanation for this frequency dependence. Instead, other constrictive neurotransmitters besides ACh may be released at high frequencies in the lower airways. High frequency stimulation (above 10 Hz) may also produce anti-dromic neurotransmitter release which may not produce physiologically relevant responses, but further study is required.

The inferior trachea frequency-response curve maximum was increased and the EF50 shifted left after pre-treatment with capsaicin and indomethacin. This is consistent with blockade of the relaxant phase of the EFS response leading to an increase in AUC. It also indicated a role for either sensory nerves, prostaglandins, or both, in causing the relaxant phase of the responses. Blocking the neuropeptide effects of substance P with CP-99994 and of CGRP with CGRP₈-₃⁷ did not produce significant changes in the
frequency response curve maximums or EF50s in the inferior trachea. However, after treatment with CGRP\textsuperscript{(8-37)} a single set of best-fit curve parameters did not describe the frequency response curves. There was a modest effect of CGRP but not substance P in the inferior trachea.

Treatment with indomethacin alone in the inferior trachea significantly changed the responses at individual frequencies and the overall shape of the best-fit curve, but altogether it did not affect the maximum or EF50 of the frequency-response curve. Because the treatment with capsaicin and indomethacin was a pre-treatment followed by a 30-minute washout period, it is difficult to know how much indomethacin was present in the tissue at the time of EFS. However, the combination of capsaicin and indomethacin appeared to produce synergistic effects when compared to the effects of capsaicin or indomethacin alone, based on the dramatic shift in EF50 and maximum of the frequency response curves. Additionally, treatment with the EP\textsubscript{2} receptor antagonist PF-04418984 produced a statistically significant leftward shift in the EF50. No effect was seen after blockade of EP\textsubscript{3} receptors with the drug L-798,106. Thus the EP\textsubscript{2} receptor is important for the relaxation phase in the inferior trachea, but not the superior trachea, while activation of EP\textsubscript{3} receptors does not appear to have a critical role in EFS-induced relaxation. The fact that EP\textsubscript{2} receptor blockade produced a significant effect, while blockade of all prostanoid formation with indomethacin did not, suggests that EFS produces multiple COX products and that some of them (e.g. PGD\textsubscript{2}, TXA\textsubscript{2}) work against PGE\textsubscript{2} to cause constriction.

The experimental results do not provide evidence for the mechanism by which CGRP causes relaxation in the lower trachea. Treatment with the NOS inhibitor L-
NOARG did not produce statistically significant effects of EFS (Appendix B, Figure 24). It is possible that generation of PGE$_2$ is the mechanism by which CGRP elicits its relaxant effects. If that is the case, PGE$_2$ must also be generated independently of CGRP, since blockade of the EP$_2$ receptor caused a more dramatic shift than blockade of CGRP alone. It is also possible that these are two independent systems. CGRP is also present in neuroepithelial bodies (NEBs) in the lungs. These specialized epithelial cells are often present at airway bifurcations (such as the inferior trachea) and are innervated. It is unknown whether these cells can be directly stimulated by EFS or if the stimulation of surrounding nerves can activate them to release chemical mediators.

In the superior trachea, only treatment with capsaicin and indomethacin and with CGRP$_{(8-37)}$ produced a change in the best-fit frequency-response curve parameters. There was no effect of pre-treatment with capsaicin and indomethacin on the maximum or EF50 of the frequency response curve. There was no detectable effect of treatment with indomethacin, CP-99994, PF-04418984 or L-798,106. No pharmacological treatment caused a significant change in the EF50 of the superior trachea. The only significant change in frequency-response was a decreased maximum after treatment with CGRP$_{(8-37)}$. This was a surprising result given the prior results from the inferior trachea. These results suggest that CGRP acts to cause constriction and that blocking it with CGRP$_{(8-37)}$ causes enhanced relaxation. If CGRP causes prostanoid formation, it may preferentially generate PGD$_2$ and/or TXA$_2$ in the superior trachea and PGE$_2$ in the inferior trachea.

The EFS methods used here can be extended to investigate neuronal control of the airways in more depth, in particular for identifying post-release mechanisms for these neurotransmitters. However, there are some limitations of these methods. The baseline
relaxation responses were weaker in the superior trachea than in the inferior trachea which makes it more difficult to measure significant changes in relaxation. AUC may not be the best measurement of responses. Analysis of response maximum or the slope of the relaxation phase may allow for more nuanced interpretations. While the EFS current did not cause any obvious damage to the tissues, it is a high voltage compared to physiological conditions. Supra-maximal voltage is needed to fully depolarize all nerves present to ensure reproducible responses. However, there is some debate as to whether EFS causes the production and release of prostanoids through physiological means or if this is an inflammatory response to the direct application of electric pulses. In guinea pigs, for instance, there is evidence that prostaglandin release is not blocked by tetrodotoxin (Fernandes, Hubbard, and Undem 1994). It is possible that NEBs or tetrodotoxin-insensitive nerves are the initiators of the prostanoid formation. Regardless of their mechanism of formation, there appear to be different quantities of prostanoids produced in the superior trachea, the inferior trachea, and in lower airways.

All studies here were on control animals without allergic or atopic asthma phenotypes. Treatment with house dust mite, cigarette smoke, electronic cigarette vapor (Appendix C), or some other model of lung disease may alter the innervation in the lung. This would be predicted to have dramatic effects on the EFS responses on ASM, and changes in their response to the drugs administered in studies such as those here may inform new therapeutic approaches for targeting nerves in airway disease. Further experiments will be needed to correlate in vitro EFS responses to neuronal control in asthma and COPD. In vivo studies will be necessary to correlate AUC of nerve stimulation in isolated airways with disease severity. Additionally, performing these
studies on human tissues would provide greater insight into how the model can be used to provide pharmacological insight in targeting nerves in asthma.

Taken together these results highlight the marked differences in innervation between the superior trachea and the inferior trachea. The PCLS results are consistent with multiple changes in airway innervation throughout the lower airways. Innervation of the lungs is heterogeneous, and future studies need to take this into account. A treatment that is effective on nerves in one part of the lung may be ineffective in another part. This is particularly relevant to keep in mind as the pharmaceutical industry moves to more big data analysis and in silico models. Modeling drug responses in one portion of the airway is likely to be very different from modeling another portion.
<table>
<thead>
<tr>
<th></th>
<th>Inferior Trachea</th>
<th>Superior Trachea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Capsaicin/Indo</td>
<td>3250 ± 409</td>
<td>5690 ± 486</td>
</tr>
<tr>
<td>CGRP(8-37)</td>
<td>4250 ± 692</td>
<td>5330 ± 707</td>
</tr>
<tr>
<td>CP999994</td>
<td>5380 ± 541</td>
<td>5250 ± 372</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5380 ± 541</td>
<td>6190 ± 247</td>
</tr>
<tr>
<td>PF-04418984</td>
<td>5750 ± 676</td>
<td>5980 ± 355</td>
</tr>
<tr>
<td>L-798,106</td>
<td>3830 ± 453</td>
<td>5040 ± 1010</td>
</tr>
</tbody>
</table>

Maximum values for the nonlinear regression of AUC results from EFS trachea constriction experiments. Mean and SEM values are taken from nonlinear regression fits in Prism. P-values are two-tailed unpaired t-tests derived from the curve maximum mean, SEM, and n calculated from nonlinear regression curves in Prism.
Table 5 - Trachea EF50s and Curve Shifts

<table>
<thead>
<tr>
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<th>Control (Hz)</th>
<th>Treatment (Hz)</th>
<th>Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin/Indo</td>
<td>8.51 (4.08, 17.7)</td>
<td>2.20 (1.20, 4.02)</td>
<td>3.88</td>
<td>0.021</td>
</tr>
<tr>
<td>CGRP (8-37)</td>
<td>7.87 (2.93, 21.1)</td>
<td>2.79 (1.11, 7.04)</td>
<td>2.82</td>
<td>0.161</td>
</tr>
<tr>
<td>CP99994</td>
<td>2.25 (1.02, 4.98)</td>
<td>1.67 (0.937, 2.96)</td>
<td>1.35</td>
<td>0.553</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2.25 (1.02, 4.98)</td>
<td>0.853 (0.623, 1.17)</td>
<td>2.64</td>
<td>0.051</td>
</tr>
<tr>
<td>PF-04418984</td>
<td>3.99 (1.59, 10.0)</td>
<td>1.23 (0.757, 2.00)</td>
<td>3.24</td>
<td>0.046</td>
</tr>
<tr>
<td>L-798,106</td>
<td>2.92 (1.09, 7.85)</td>
<td>7.72 (1.58, 37.8)</td>
<td>2.65</td>
<td>0.324</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control (Hz)</th>
<th>Treatment (Hz)</th>
<th>Fold Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin/Indo</td>
<td>2.80 (2.13, 3.68)</td>
<td>2.01 (1.40, 2.88)</td>
<td>1.39</td>
<td>0.168</td>
</tr>
<tr>
<td>CGRP (8-37)</td>
<td>3.55 (2.32, 5.43)</td>
<td>3.36 (2.30, 4.91)</td>
<td>1.06</td>
<td>0.414</td>
</tr>
<tr>
<td>CP99994</td>
<td>2.97 (0.679, 13.0)</td>
<td>6.83 (1.42, 32.9)</td>
<td>2.30</td>
<td>0.462</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2.97 (0.679, 13.0)</td>
<td>3.79 (0.915, 15.7)</td>
<td>1.28</td>
<td>0.817</td>
</tr>
<tr>
<td>PF-04418984</td>
<td>1.48 (0.802, 2.71)</td>
<td>2.20 (0.782, 6.18)</td>
<td>1.49</td>
<td>0.522</td>
</tr>
<tr>
<td>L-798,106</td>
<td>1.87 (1.02, 3.45)</td>
<td>4.99 (1.50, 16.6)</td>
<td>2.67</td>
<td>0.190</td>
</tr>
</tbody>
</table>

Tables for the EF50 results from EFS trachea constriction experiments in (A) inferior and (B) superior tracheas. EF50 values are given in arithmetic units with 95% confidence intervals in parentheses. P-values are two-tailed unpaired t-tests derived from the logEF50 mean, SEM, and n calculated from nonlinear regression curves in Prism.
CHAPTER 4: EXTENSION OF THE COMPUTATIONAL MODEL

Introduction

Chapter 2 described the development of a computational model to predict the behavior of tracheas in response to EFS. These models were developed exclusively using data taken from the inferior trachea. As highlighted in Chapter 3, there are profound distributional differences in neurotransmitter effects in different portions of the airways. The inferior trachea has a profound relaxation component, while the relaxation phase of the superior trachea is less pronounced. In this final chapter, two extensions of the models described in Chapter 2 are presented. First, the First-Order and the Inhibition models are applied to the control and capsaicin/indomethacin treated superior trachea data. Second, using the pharmacological results from the previous chapter, PGE$_2$ activation of the EP$_2$ receptor is incorporated into the model as a second relaxation component of the inferior trachea control data. The addition of PGE$_2$ improves the model fit to control data and accurately predicts the rapid relaxation phase of the inferior trachea. The chapter concludes with final thoughts about further improvements to the model and its applications moving forward.

Methods

Superior Trachea Model

Methods for EFS stimulation and subsequent modeling are described in detail in Chapter 2. Briefly, CF-1 mice were sacrificed and the superior portion of the trachea closest to the larynx was isolated. Each trachea was suspended in an isolated tissue bath on a 3D-printed post with platinum wire electrodes on either side of the tissue. The force of contraction was measured. EFS was applied at half-log$_{10}$ frequencies from 0.1 Hz to 10
Hz. Tracheas were then pre-treated with 100 μM capsaicin and 10 μM indomethacin for 2 hours followed by a one-hour washout period. After washout a second EFS frequency response curve was generated.

The models were designed using the RxODE package in R to solve the systems of differential equations that describe the model given initial parameters and a drug administration regimen (Wang, Hallow, and James 2016). Both the First-Order and Inhibition models were applied to the superior trachea data. Each model was applied to the data from the control and the Treated tissue constriction experiments. A least sum of squares method was used to find best-fit values for the $k_a$, $k_e$, and pDose values in the First-Order model. The same method was used to find $M_{2\text{max}}$, $ACh_{\text{max}}$, $pIC_{50M2}$ and $pIC_{50AChE}$ best-fit parameters in the Inhibition model.

Multiple Neurotransmitter Model

Results from the isolated trachea tissue bath experiments from Chapter 3 were used to inform improvements to a computational model. In previous experiments, inferior tracheas were treated with capsaicin and indomethacin to block sensory neuronal activity and prostaglandin formation, respectively, in EFS experiments. Together these drugs isolated the ACh components of EFS and allowing for a one compartment distribution model to accurately predict the data. However, ACh alone was not sufficient to explain the relaxation phases in the EFS responses. Identification of $PGE_2$ as a key relaxant mediator in the inferior trachea (see Chapter 3) informed improvements to the model. $PGE_2$ activation of EP$_2$ receptors was added as a second relaxant neurotransmitter to the ACh-only Inhibition model. Control results, whose ACh kinetic parameters were solved for in Chapter 2, were modeled using a $PGE_2$ EC50 value ($1.2 \times 10^{-8}$ M) consistent with
literature values (Michitaka et al. 1997). In the model, PGE$_2$ is released alongside ACh at each pulse of EFS. A First-Order model (Chapter 2, Equations 1 and 2) governed by the first order absorption ($k_a$) and elimination ($k_e$) rate constants and a dose of PGE$_2$ administered after each pulse (pDose) were used. The effect of PGE$_2$ decreased the maximum contractile effect of ACh (Equation 6).

$$
Effect = \left( E_{max,ACh} - \frac{C_{1,PGE2}}{EC50_{PGE2} + C_{1,PGE2}} \right) \frac{C_{1,ACh}}{EC50_{ACh} + C_{1,ACh}}
$$

(6)

**Statistics**

Modeling, parameter fitting, plotting, and general statistics were performed with R (3.5.0) (R Core Team 2017) using the RStudio graphical user interface (RStudio Team 2016). Comparison tests were unpaired two-tailed t-tests and a p-value less than 0.05 was considered statistically significant. Data are presented as means ± SEMs. All R code used for fitting the models and for analysis can be found at https://github.com/CamKieff/NervePKPDmodel.

**Results**

**Superior Trachea Model Fits**

Frequency-specific results for the superior trachea model fits were different in key ways from the inferior trachea model fits. Unlike the control First-Order fits for the inferior trachea, the pDose value did not appear to increase as frequency increased. The pDose at 1 Hz ($5.82 \pm 0.03$) was the only frequency that was significantly different from the others (Table 6A). Also, unlike the inferior control First-Order fits, the $k_a$ at 0.1 Hz was significantly larger than at any of the other frequencies ($2.00 \pm 0.89$) but had a large standard error (Table 6A). This is most similar to the results from the inferior capsaicin-
and indomethacin-treated First-Order fit, which also displayed a larger $k_a$ at 0.1 Hz (Chapter 2, Table 3). The $k_e$ fits were not significantly different across frequencies.

Like the superior control tissue First-Order and the inferior Treated tissue First-Order best fits, the superior Treated tissue First-Order model produced a $k_a$ that was significantly larger at 0.1 Hz than all the other frequencies at $2.67 \pm 0.19$ (Table 7A). Again, $k_e$ did not vary with frequency. The pDose values were lower ($5.69 \pm 0.08$) on average at 1 Hz than at any of the lower frequencies. Additionally, the pDose at 10 Hz was lower ($5.57 \pm 0.09$) than the pDose found at 0.1, 0.3, and 3 Hz (Table 7A).

For the Inhibition model of the superior trachea control response, there were no significant differences in $M_{2max}$, $AChE_{max}$, $pIC50_{M2}$ or $pIC50_{AChE}$ best-fit parameters. This may be due to large variability between tissues for some of the parameters, particularly $AChE_{max}$ (Table 6B). However, there were significant differences in the capsaicin/indomethacin-treated superior trachea Inhibition model results. The $pIC50_{AChE}$ was higher than all other frequencies at 1 Hz ($8.60 \pm 0.97$, Table 7B). The $pIC50_{M2}$ parameter was frequency dependent, going from an average of $6.99 \pm 0.08$ at 0.3 Hz to a value of $5.89 \pm 0.06$ at 10 Hz (Table 7B). This is consistent with the trend seen in the $pIC50_{M2}$ results from the Treated tissue Inhibition best-fit in the inferior trachea.
Table 6 - Superior Trachea Control Best-Fit Parameter Results

A

<table>
<thead>
<tr>
<th>Hz</th>
<th>$k_a$</th>
<th>$k_c$</th>
<th>pDose</th>
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<tbody>
<tr>
<td>0.1</td>
<td>2.00 ± 0.89</td>
<td>0.94 ± 0.21</td>
<td>6.07 ± 0.07</td>
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<tr>
<td>0.3</td>
<td>0.30 ± 0.02*</td>
<td>0.87 ± 0.05</td>
<td>6.00 ± 0.001</td>
</tr>
<tr>
<td>1</td>
<td>0.19 ± 0.02*</td>
<td>0.76 ± 0.03</td>
<td>5.82 ± 0.03* &amp;</td>
</tr>
<tr>
<td>3</td>
<td>0.36 ± 0.09*</td>
<td>0.85 ± 0.04</td>
<td>6.00 ± 0.00#</td>
</tr>
<tr>
<td>10</td>
<td>0.19 ± 0.04*</td>
<td>0.79 ± 0.12</td>
<td>5.96 ± 0.03#</td>
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B

<table>
<thead>
<tr>
<th>Hz</th>
<th>M2_{max}</th>
<th>AChE_{max}</th>
<th>pIC50_{M2}</th>
<th>pIC50_{AChE}</th>
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</thead>
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<tr>
<td>0.3</td>
<td>0.99 ± 0.01</td>
<td>0.35 ± 0.09</td>
<td>6.65 ± 0.24</td>
<td>6.69 ± 1.07</td>
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<tr>
<td>1</td>
<td>0.54 ± 0.25</td>
<td>0.55 ± 0.07</td>
<td>5.40 ± 1.52</td>
<td>8.01 ± 1.07</td>
</tr>
<tr>
<td>3</td>
<td>0.88 ± 0.05</td>
<td>0.34 ± 0.09</td>
<td>6.24 ± 0.36</td>
<td>4.75 ± 1.33</td>
</tr>
<tr>
<td>10</td>
<td>0.91 ± 0.04</td>
<td>0.49 ± 0.12</td>
<td>6.74 ± 1.00</td>
<td>6.26 ± 1.07</td>
</tr>
</tbody>
</table>

Results from the (A) First-Order and (B) Inhibition models in the control superior trachea. Data are means ± SEMs (n = 5). The asterisk (*) indicates a statistically significant difference (p-value < 0.05) from 0.1 Hz parameter using a one-way ANOVA with a Tukey post-test. A significant difference from 0.3 Hz, &; significant difference from 1 Hz, #; significant different from 3 Hz, $.
Table 7 - Superior Trachea Treated Best-Fit Parameter Results

<table>
<thead>
<tr>
<th></th>
<th>Hz</th>
<th>kₐ</th>
<th>kₑ</th>
<th>pDose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>2.67 ± 0.19</td>
<td>0.80 ± 0.07</td>
<td>5.94 ± 0.05</td>
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<tr>
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<td>0.74 ± 0.05</td>
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<td>5.69 ± 0.08*</td>
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<td>0.70 ± 0.02</td>
<td>5.57 ± 0.09* &amp; $</td>
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<th>Hz</th>
<th>M₂max</th>
<th>AChE_max</th>
<th>pIC50_M₂</th>
<th>pIC50_AChE</th>
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<tr>
<td>B</td>
<td>0.3</td>
<td>0.99 ± 0.01</td>
<td>0.49 ± 0.14</td>
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<td>4.61 ± 1.30</td>
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<td>1</td>
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<td>0.53 ± 0.02</td>
<td>6.62 ± 0.06 &amp;</td>
<td>8.60 ± 0.97 &amp;</td>
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<td>0.76 ± 0.05</td>
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<td>4.77 ± 0.22 #</td>
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<td>0.98 ± 0.003 &amp;</td>
<td>0.83 ± 0.09 &amp;</td>
<td>5.90 ± 0.06 &amp; # &amp; $</td>
<td>5.05 ± 0.09 #</td>
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</tbody>
</table>

Results from the (A) First-Order and (B) Inhibition models in the superior trachea after pre-treatment with capsaicin and indomethacin. Data are means ± SEMs (n = 5). The asterisk (*) indicates a statistically significant difference (p-value < 0.05) from 0.1 Hz parameter using a one-way ANOVA with a Tukey post-test. A significant difference from 0.3 Hz, &; significant difference from 1 Hz, #; significant different from 3 Hz, $.
Like the inferior trachea results, the results here should be applicable across all frequencies. The only difference between frequencies should be the time elapsed between doses; the kinetics of the mediators should stay the same. The median responses for each frequency were averaged for each tissue individually and those parameters were applied to the model to predict the tissue responses at every frequency. In the superior trachea, the mean AUC of the tissue-specific all-frequency fits for the control First-Order model was only significantly lower than the experimental data at the 1 Hz frequency, while the complex model was also significantly lower at 1 Hz but was significantly higher at 3 Hz (Table 8A). Both the First-Order and the Inhibition model AUCs of the control data in the inferior trachea were significantly different at 3 frequencies (Table 8A). Conversely, the Treated superior data displayed no significant AUC differences between either the First-Order or Inhibition model best fits. The First-Order and Inhibition model fits also displayed no AUC differences in the inferior trachea.

In addition to AUC, the ability of the superior trachea model to predict maximum responses to EFS were evaluated. Unlike the AUC, the models did not readily calculate the maximum effect. Every predicted maximum of the control superior trachea response using the First-Order model was significantly lower than the experimental response (Table 8B). The Inhibition model provided a better fit, as it accurately predicted maximum control contractions to EFS at the two highest frequencies, 3 and 10 Hz, but also predicted lower values for the lower frequencies. In the Treated tissue data, the models predictably fared better. The First-Order model was conservative in its maximum predictions for the Treated superior responses at 0.1, 3, and 10 Hz (Table 8B). The Inhibition model correctly predicted the maximal response at all frequencies except for
0.1 Hz, where, again, it was too low (Table 8B). Like the inferior trachea, the Inhibition model made the most accurate predictions when modeling Treated tissues with a single ACh-induced constriction phase.
Figure 16 - Control Superior Trachea Distribution Model Results

Representative plot of control model results for superior tissue 2. Each panel represents a different frequency from 0.1 to 10 Hz. The three datasets plotted in each panel are the normalized experimental data tracing (grey), the First-Order model fit (blue), and the Inhibition model fit (red). Each response is normalized to the maximum tissue EFS response (30 Hz) in the presence of capsaicin and indomethacin.
Representative plot of superior trachea response after pre-treatment with capsaicin and indomethacin. Each panel represents a different frequency from 0.1 to 10 Hz. The three datasets plotted in each panel are the normalized experimental data tracing (grey), the First-Order model fit (blue), and the Inhibition model fit (red). Each response is normalized to the maximum tissue EFS response (30 Hz) in the presence of capsaicin and indomethacin.

Figure 17 - Treated Superior Trachea Distribution Model Results
Table 8 – AUC and Maximum Results for the Superior Trachea

A

<table>
<thead>
<tr>
<th></th>
<th>Capsaicin</th>
<th>0.1 Hz</th>
<th>0.3 Hz</th>
<th>1 Hz</th>
<th>3 Hz</th>
<th>10 Hz</th>
</tr>
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<tr>
<td>Raw</td>
<td>-</td>
<td>1.80 ± 0.27</td>
<td>4.51 ± 0.23</td>
<td>18.0 ± 0.43</td>
<td>26.2 ± 0.51</td>
<td>43.6 ± 1.53</td>
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<tr>
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<td>+</td>
<td>2.53 ± 0.18</td>
<td>6.09 ± 0.44</td>
<td>21.9 ± 1.80</td>
<td>30.2 ± 1.53</td>
<td>50.6 ± 1.03</td>
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<tr>
<td>First-Order</td>
<td>-</td>
<td>1.88 ± 0.13</td>
<td>4.95 ± 0.22</td>
<td>13.6 ± 0.48*</td>
<td>27.7 ± 0.66</td>
<td>44.0 ± 0.49</td>
</tr>
<tr>
<td>First-Order</td>
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<td>2.82 ± 0.26</td>
<td>7.81 ± 0.68</td>
<td>19.6 ± 1.38</td>
<td>35.0 ± 1.61</td>
<td>48.9 ± 1.03</td>
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<tr>
<td>Inhibition</td>
<td>-</td>
<td>1.95 ± 0.29</td>
<td>5.43 ± 0.47</td>
<td>15.7 ± 0.69*</td>
<td>30.6 ± 0.77*</td>
<td>45.7 ± 0.58</td>
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<tr>
<td>Inhibition</td>
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<td>2.53 ± 0.32</td>
<td>7.85 ± 1.16</td>
<td>21.0 ± 2.82</td>
<td>34.3 ± 2.62</td>
<td>47.3 ± 1.31</td>
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</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Capsaicin</th>
<th>0.1 Hz</th>
<th>0.3 Hz</th>
<th>1 Hz</th>
<th>3 Hz</th>
<th>10 Hz</th>
</tr>
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<tbody>
<tr>
<td>Raw</td>
<td>-</td>
<td>0.13 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.38 ± 0.01</td>
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<td>0.17 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.44 ± 0.04</td>
<td>0.58 ± 0.03</td>
<td>0.94 ± 0.02</td>
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<tr>
<td>First-Order</td>
<td>-</td>
<td>0.07 ± 0.01*</td>
<td>0.10 ± 0.004*</td>
<td>0.24 ± 0.01*</td>
<td>0.48 ± 0.01*</td>
<td>0.76 ± 0.01*</td>
</tr>
<tr>
<td>First-Order</td>
<td>+</td>
<td>0.12 ± 0.01*</td>
<td>0.16 ± 0.01</td>
<td>0.34 ± 0.02*</td>
<td>0.60 ± 0.03</td>
<td>0.83 ± 0.02*</td>
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<tr>
<td>Inhibition</td>
<td>-</td>
<td>0.08 ± 0.01*</td>
<td>0.11 ± 0.01*</td>
<td>0.28 ± 0.01*</td>
<td>0.55 ± 0.01</td>
<td>0.81 ± 0.01</td>
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<tr>
<td>Inhibition</td>
<td>+</td>
<td>0.13 ± 0.01*</td>
<td>0.16 ± 0.02</td>
<td>0.38 ± 0.05</td>
<td>0.67 ± 0.04</td>
<td>0.89 ± 0.01</td>
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</table>

Tables showing (A) AUC and (B) maximum results for raw trachea constriction data, First-Order model and Inhibition model best fits. The asterisk (*) indicates a difference between model fit and corresponding raw data. (p < 0.05; two-tailed unpaired t-test).
Multiple Neurotransmitter Model Fits

Single-run, best-fit curves for the four control interior trachea responses are shown in Figure 18. These are the same control responses used to develop the models in Chapter 3. AUC results for frequencies at 1 Hz and above were more accurate than either the First-Order or Inhibition model with ACh alone. In these results, the Multiple Neurotransmitter model successfully predicted a relaxation phase, and this phase terminated near the plateau reached in the experimental recording. The relaxation phase in tissue 4 was very mild. The shallow slope of the reaction was like the relaxation seen in the superior trachea tissues. At 10 Hz the model did not predict a slow relaxation phase. This may indicate that the model as presented would have difficulty predicting superior trachea responses without some improvements.

Unlike the inferior and superior single neurotransmitter models, it was not possible to find a single set of parameters for PGE$_2$ that accurately reflected the experimental tracings across all frequencies. In fact, even averaging the parameters between model runs produced combinations of parameters that did not predict the relaxation phases. Therefore, it will be necessary to determine a new method for constructing a relaxation model that applies across frequencies. There is a frequency dependence in the relaxation phase that is not captured in the model. This prevented in-depth statistical analysis of the predicted model results.
Figure 18 - Two Drug Model Initial Results

Plots of single model runs that incorporate PGE$_2$ activity into the ACh Inhibition model. The raw control data is plotted in grey while the model results are plotted in red. Each row represents a different tissue from the inferior trachea, the same trachea used in the model development in Chapter 2. Each panel represents a different frequency, either 1, 3, or 10 Hz. Each response is normalized to the maximum tissue EFS response (30 Hz) in the presence of capsaicin and indomethacin.

Discussion

In general, because of the less pronounced relaxation phase, both the First-Order model and the Inhibition model fitted the control data better in the superior trachea than in the inferior trachea. After treatment with capsaicin and indomethacin, the shape of the EFS curves were similar between the inferior and the superior trachea. In these Treated tissues, the model fits were of similar quality. Both the superior and inferior Inhibition models had frequency-dependent best-fits for the pIC$_{50}$M$_2$ parameter. This may indicate additional missing components in the functional form of the differential equations used to describe the system.

The relaxation phases in the superior trachea were less dramatic than in the inferior trachea, so, intuitively, the ACh-only models should predict control responses better in the superior trachea compared to the inferior trachea. This is indeed what was observed. In the inferior trachea, both the First-Order and Inhibition models predicted significantly different AUCs at three frequencies (Chapter 2, Table 2). In the superior trachea, these numbers were reduced to two erroneous AUC predictions in the Inhibition model and only one erroneous AUC prediction in the First-Order model (Table 8A). In
both trachea types, the Inhibition and First-Order models predicted AUC responses that were not significantly different from the capsaicin- and indomethacin-treated trachea constriction responses.

The lowest frequencies of neurotransmitter release, 0.1 and 0.3 Hz, may be the most physiologically relevant. However, there was not much benefit in plotting 0.1 and 0.3 Hz model fits for the Multiple Neurotransmitter model. AUC results were comparable to those in the ACh-only Inhibition model. Applying the averaging techniques to find a single set of best-fit PGE$_2$ parameters that accurately modeled the experimental data at all frequencies was not possible using the methods from the ACh-only model. There could be additional regulatory mechanisms at higher concentrations that need to be incorporated into the model, similar to how the Inhibition model builds on the First-Order model. It is also possible (and consistent with experimental results in Chapter 3) that there are multiple relaxation mediators released during EFS, and that these alter the kinetics of the relaxation phase.

An alternative hypothesis is that the method of averaging parameters across frequencies is not the most efficient method for determining frequency-spanning best-fit parameters. It may be better to fit the model to all frequencies at the same time. The challenge in this method is finding an objective function to minimize. Least sum of squares attempts to minimize the squared absolute errors. This favors over-fitting of the higher frequency responses because their absolute values are larger. Another approach would be to run the model even more times and simply select the set of parameters that give the lowest amount of error.
The Multiple Neurotransmitter model does not assume independence between the activities of PGE\(_2\) and ACh on ASM. Instead, PGE\(_2\) directly inhibits the maximum ACh effects on ASM. This is analogous to bronchodilation by G\(\alpha_s\) antagonizing G\(\alpha_q\)-induced bronchoconstriction. While the effects of the neurotransmitters are not independent, their kinetics are. Neither ACh nor PGE\(_2\) act in the model to promote or inhibit the first order rate constants \(k_a\) or \(k_e\) of the other chemical; they also fail to modulate the pDose released at each pulse. The frequency dependence seen in the PGE\(_2\) component could be due to ACh-mediated inhibition of PGE\(_2\) formation.

The First-Order, Inhibition, and Multiple Neurotransmitter models are all proofs of concept that it is possible to model the interaction between the nerves and the airways. More work is needed to ensure the models can be correlated with in vivo disease states. Specifically, an electronic cigarette exposure assay is being developed for mice (Appendix C). This will allow for analysis of the effects of e-cigarettes on neurotransmitter release in EFS. As discussed earlier, isolated tissue experiments have benefits over cell-based experimental systems because the interactions between the airways, nervous system, and immune system can be studied together. Bachar et al. (2004) have used a similar isolated trachea EFS system to study interactions between nerves and the immune system. Another interesting method to study these complex systems is presented by Voedisch et al. (2012). They used imaging of live lung slices and measured the movement of fluorescent dendritic cells in response to EFS.

The development of lung-on-a-chip technology (Huh et al. 2010) could provide an organ level high-throughput system for the study of lung disease. These chips are even being used to study the effects of cigarette smoke and the development of COPD (Benam
et al. 2016). Unfortunately, the models do not currently incorporate nerves or organism-level immune functions. Improved models based on the ones presented here could be incorporated into high-throughput data from lung-on-a-chip experiments. Together, these data could more accurately make predictions of the effects of drugs *in vivo*.

**Conclusions**

In the work presented here, I have established a system for testing the effects of EFS on isolated mouse trachea using a custom, 3D-printed post. I demonstrated that the contractions in response to EFS were cholinergic in nature. I explored and characterized the differences in responses to EFS between the superior trachea, inferior trachea, and lower airways. I confirmed the important role of PGE$_2$ in causing relaxation of isolated trachea after EFS. I developed a mathematical distribution model that accurately predicts inferior trachea constriction responses to EFS. Furthermore, I extended that model to the functionally distinct superior part of the trachea and incorporated PGE$_2$ as a second EFS-activated chemical mediator.

Taken together, this work contributes a novel perspective to the study of the functional neurotransmitter systems in the lung. Expansions of the projects presented here will allow for the *in vitro* study of the interactions between the airways, the nervous system, and the immune system that would not be possible in cell- or chip-based systems. Additionally, models like the ones presented here may aid in the *in-silico* testing of drugs, furthering the development of new therapeutics for the treatment of asthma and other airway diseases.
APPENDIX A: DISTRIBUTION MODEL DESCRIPTION

This repository for the code can be accessed at
https://github.com/CamKieff/NervePKPDmodel.

The raw data are contained in two folders corresponding to the upper trachea and
the lower trachea. Both folders are subdivided into control (con) and capsaicin (cap)
folders. Data are CSV files formatted with “Time” in the far-left hand column followed
by increasing frequency response data from “0.1Hz” to “30Hz”. The data are trachea
responses measured in mg of tension. Time is in seconds with measurements every 0.02
seconds. Total record time is 60 seconds. All data have been formatted so that EFS
begins at Time = 0. Data in the capsaicin folder has been pre-treated for two hours with
100 μM capsaicin and 10 μM indomethacin followed by a one-hour washout period. In
the inferior trachea files 1, 2, 5, and 7 had characteristic responses and were used in
analysis. Data files 1, 2, 3, 4, 5 were all used in the superior trachea analysis.

NB: In the code, the First-Order model is referred to as the “simple” model and the
Inhibition model is referred to as the “complex” model. These terms are used in this
description rather than the names given in Chapter 2.

**do.R and do_sup.R**

The file do.R imports the necessary functions and the appropriate data and runs
the model. Running this file will duplicate the approach I used to find the best fit
parameters for the control and capsaicin inferior trachea data. It will produce the output
files that can be used in conjunction with the consensus_plots.R file to make plots and to
find the aggregate parameters across frequencies and tissues.
First it establishes a naive set of initial parameters and defines the First-Order model. Next it solves the model 100 times for the 0.1 Hz frequency and then for the 0.3, 1, 3, 10 Hz frequencies for both the capsaicin and control data. The 0.1 Hz frequencies are solved separately because the experimental stimulation frequency is slightly different from 0.1 Hz and this difference can dramatically affect the fit. As such the model fits the Frequency at 0.1 Hz in addition to the other parameters.

The 0.1 Hz best fit is also used for the $k_a$, $k_c$, and pDose values for the complex models. This model is solved next for both capsaicin and control. The final parameters for each tissue are averaged to determine a single set of best fit parameters for each tissue and treatment. Finally, it creates a second file with the results broken down by frequency rather than by tissue.

The file do_sup.R follows the same code, but performs it for the superior rather than the inferior trachea.

**model_2drugs.R**

This is a working file that contains instances of running the model functions described below. The model workflow begins with loading required packages and files, then the initial model parameters are defined, the selected pharmacokinetic model is compiled, and the data file to be tested is imported. The model is run to find best-fit parameters and the results can be exported or graphed. This file gives an idea of how the functions can be used to solve for parameters and plot fits.
normalizedDF.R

The function `loadNormalizedDF` imports a selected data frame and normalizes the mg of tension response to a maximum. Parameters passed to the function include the section of trachea (lower = TRUE or FALSE) where the data was collected, data to be normalized (“con” or “cap” data), data to be used in the denominator to normalize (again, “con” or “cap” data), and the index for the file in its respective folder (integer that indicates the position of the file in the folder). The function finds the maximum value and minimum values in the normalizing data and uses the difference as the scaling factor. This is frequently, but not always, the response at 30 Hz. In the lower trachea, capsaicin data is used to normalize both capsaicin and control data. In the upper trachea however, control responses may have greater maxima than capsaicin responses and it may be necessary to normalize control responses to themselves.

defineModel.R

The `defineModel` function from defineModel.R creates and compiles the model to be tested using the `RxODE` package. The constrictive ACh component of the model can be the First-Order model (“simple”) with first-order one-compartment kinetics or the Inhibition model (“complex”) with saturable kinetics for absorption and elimination. It is possible to create models with multiple neurotransmitters using this function. The unknown relaxant component can be non-existent (“none”) or present as a one-compartment first-order neurotransmitter (“simple”). If there is no unknown relaxant the effect model will default to a single neurotransmitter effect (pharmacodynamic) model. If there is a defined unknown relaxant model there are two options for the way these two
neurotransmitter effects are summed together. In the case of the “twoNT_1” option the relaxant effect is subtracted from the ACh effect and the product of the two effects is added back, analogous to the Bliss Independence model. In the “twoNT_2” option the unknown effect is instead subtracted from the ACh maximum effect. The function returns an object that contains the model, the model’s initial conditions, and the number of neurotransmitters in the model. The information from this object is read by subsequent functions.

This function allows for the rapid selection from 6 relevant model choices using one line of code. Of course, other models can be used, but these will have to be developed individually and have not been validated to work with the rest of the code presented here.

runModelFunctions.R

This file contains the major workhorses that bring the magic of the model together, the run_mod1 and final_drug_params functions, as well as several other useful functions. After importing the data, defining a model, and choosing a set of initial parameters, run_mod1 solves the model using the RxODE package.

run_mod1 first maps the “init_params” to the parameters defined in the model. Next it builds the event table. The event table is a list of doses of drug to be administered based on the EFS frequency. Finally, it solves the model and returns a data frame of the compartment concentrations and predicted effects at each time point (every 0.02 s).

One run of final_drug_params finds a set of best-fit parameters for the chosen model. The function runs run_mod1 repeatedly, each time choosing new parameters and
trying to minimize the sum of squares (Figure 19). The parameters to be optimized are defined in the “bestfit” vector. Any combination of parameters from the “init_params” list can be fit by the function if they are in the defined model. The results of `final_drug_params` can return either a data frame of either all the tested parameters (chosen = FALSE) or a data frame of only the tested parameters that were accepted as improved fits for the model (chosen = TRUE). In either case, the final row of the returned data frame are the best-fit parameters for that run.

The default number of iterations to find the best fit (“m”) is 50 for rapid testing purposes, but 500 is more appropriate to ensure the model converges. Two hyper-parameters (“lambda” and “testexp”) can also adjust the rate of convergence. The width (standard deviation) of the Gaussian curves used to sample new best-fit parameters is proportional to lambda. A larger “lambda” will reduce the influence of the initial parameters by searching a larger parameter space but may also waste many iterations by attempting numbers that have little chance of improving the fit. By trial and error, 0.1 works well. The “testexp” hyper-parameter changes the penalty for having large variations from the experimental data. The larger “testexp” is the more stringent the best-fit criteria are but should always be an even integer (2 or 4 are recommended). The function `final_drug_params` also imposes some hard boundaries on a few of the parameters. AChE_{max} and M2_{max} and not allowed to go above 1. The parameters pIC50_{AChE} and pIC50_{M2} are constrained to less than 10.
**Figure 19 - Least Sum of Squares Diagram**

Visual representation of the workflow for the method of least squares. For more information of the implementation of this method, see the methods section in Chapter 3.
After the `final_drug_params` function has been run `run_mod1` is run again on the final set of parameters to generate concentration vs. time data to be plotted. An example of plotting the resulting data exists in `model_2drugs.R` using the `ggplot2` R package (Wickman, 2016). The example plotting function given plots the experimental data in black, initial results of `run_mod1` in violet, and the final best-fit results in blue.

`Iteration` is a wrapper function that takes a list of file indices (`con_list`) and some normalization inputs and generates n sets of final best-fit parameters for each file at each frequency (`freq_list`) using the defined model (ITmodel). The default value of n used in most runs was 100. After each frequency it appends its results to a single CSV file per index. These files can be reimported later to calculate their statistics.

`facetgraph` is another wrapper function that produces a single multi-panel graph using `ggplot2` where each panel is a different frequency for the same model parameters. If `consensus = FALSE`, the `init_params` are taken as a starting point and `final_drug_params` is run a single time at each frequency. This was useful for the Multiple Neurotransmitter model (Chapter 4, Figure 18). The resulting plot displays the experimental data in black, initial parameter results in blue, and the final best-fit results in red. Alternatively if `consensus = TRUE`, it will instead assume the “init_params” are the final consensus values and not run `final_drug_params`. Users can also supply `consensus_params` to plot a second set of values in the facet graph (All the 5 panel plots of the First-Order and Inhibition model

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use this methodology). This is good for producing result graphs or for visual comparison of a model’s fit at all frequencies.

The aggregate_stats function imports the results from Iteration and finds the mean, median and standard deviation (sd) for each file given in the index by frequency. It exports them to a new file given by the output_filename.

The final function in runModelFunctions.R is find_allfreq_params. This was developed after most of the data for the dissertation was generated. Instead of finding best fit values for a single frequency like final_drug_params, it finds best fit values for all frequencies at once. The objective function it tries to minimize is the sum of the sum of squares/AUC. This still needs to be tested more rigorously but could be a way to improve the fits of the model.

consensusPlot.R

consensusPlot.R is similar to model_drugs.R because it is a rough staging ground for running a collection of the functions discusses above. It uses the facetgraph function, as well as finds the AUC, sums of squares (SS) and max results. It takes finalparameters.csv as input and can create plots using the facetgraph function. Each AUC, SS, and max data frame needs to be found manually and appended to the appropriate data frame. After they have all been run, then the results can be exported. This code is not completely organized and should be used with caution.
APPENDIX B: SUPPLEMENTAL FIGURES

Figure 20 - The First-Order Model Predicts Treated Data After Least Sum of Squares Fit

Example fit of the First-Order model at 0.1 Hz of Treated tissue data. Grey is a 0.1 Hz tracing for Tissue 1. The violet curve is the naive initial parameters for the model. The blue curve is the best fit for the First-Order model after applying the method of least squares. The method of least squares effectively finds a set of parameters that improves the model fit of the experimental data. The First-Order model can fit data at low frequencies.
Figure 21 - Trachea ACh Dose Response Curves

Nonlinear dose response curves in response to ACh. The inferior (n = 6) and superior (n = 4) trachea responses could not be fit by a single set of parameters (F = 11.9, p < 0.0001). The logEC50 for the superior trachea (5.383) was used in the model curve fits because it had less influence from non-ACh mediators.
Figure 22 - PCLS Perfusion Chamber

Diagram of PCLS perfusion chamber (RC-27NE2, Warner Instruments, Hamden, CT).

HBSS is infused from the right side. The PCLS floats, it is necessary to weigh it down with a Teflon ring or piece of plastic tubing. Two heating elements are controlled externally by an automatic temperature controller (TC-344B, Warner Instruments, Hamden, CT). On either side of the slice are two platinum wire electrodes which pass current over the slice. These electrodes are connected to a Grass S44 stimulator. The slice should not contact the wires during EFS.
Figure 23 – Trachea MgSO₄ Treatment EFS Responses

Frequency-response curves in the inferior (left) and superior (right) tracheas to treatment with 10 mM MgSO₄ (n = 3, 3). Responses are AUC normalized to maximal KCl constriction. Points are means ± SEMs. *, p < 0.05 (two-tailed paired t-tests)
**Figure 24 - L-NOARG Inferior Trachea EFS Responses**

Frequency response curve for the inferior trachea in the presence and absence of the nitric oxide synthase inhibitor L-NOARG (200 nM, n = 2). Responses are AUC normalized to maximum KCl constriction. Points are means ± SEMs.
APPENDIX C: ELECTRONIC CIGARETTES

Introduction

Cigarette use in the United States peaked in the 1960s when 50% of men and 33% of women smoked. The rates of smoking have since decreased, but smoking continues to kill more than 480,000 people in the US each year. Smoking increases the risk of heart disease, stroke, and causes 90% of all lung cancers.

The first electronic cigarette (commonly referred to as an e-cigarette or simply “e-cig”) was developed in 2003 and became available in the United States around 2006. This novel, battery-operated nicotine delivery system is marketed as a less toxic way to smoke and as a smoking cessation aid. However recent studies have identified formaldehyde, a known carcinogen, as well as other trace toxins in e-cigarette vapor. More research is needed to understand both the acute and chronic effects of e-cigarettes.

The Arduino Uno is an open-source microcontroller developed in Italy. It was designed to be a simple to use electronic prototyping platform for use by non-engineers. It is a robust system that is easily customizable and, at under $25, it is ideal for use in labs with limited budgets. The purpose of the present study was to establish a cost-effective system for administering e-cigarette vapor to animals and for testing the effects of acute e-cigarette exposure in a mouse model using the Arduino microcontroller.

Methods

Experiments using animals were approved by Creighton University’s Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.
The Arduino microcontroller board was wired to a diaphragm pump. A simple Arduino script created using the Arduino Integrated Development Environment (IDE) controls the pump by closing a circuit via an N-Type MOSFET transistor. By opening and closing the transistor, the Arduino in-effect can turn the pump on and off. Originally the pump control circuit was soldered on a prototyping board (Figure 25C), but eventually the components were consolidated onto a custom-soldered shield that compactly sits on top of the Arduino circuit board (Figure 25D). The full circuit diagram is given in Figure 26. Via the Arduino IDE the pump activation time can be controlled. In these experiments the diaphragm pump was activated for two seconds every minute. Tubes connected the pump to a small animal chamber and to the e-cigarette. The pump was calibrated to deliver a flow rate of 1.1 L/min, the FTC standard.

The Vuse electronic cigarette (R.J. Reynolds Vapor Company, Winston-Salem, NC) was used in these experiments. At the time, Vuse was the most popular e-cigarette on the market with 33% market share. The e-cigarette “juice” contains approximately 4.8% nicotine suspended in a mixture of glycerin and propylene glycol.

Female CF-1 mice over six weeks in age were administered an open field test for 30 minutes before being placed in a small chamber where they were exposed to vapor from a Vuse e-cigarette (“vaped”) for one hour. The vaping was administered by an Arduino controlled diaphragm pump (TD-3LSA(19), Brailsford & Co, Antrim, NH) connected to the Vuse e-cigarette. Occasionally the e-cigarette cartridge would have to be replaced in the middle of the treatment. Control mice were placed in isolation for one hour. After exposure, a second open field test was administered to each mouse for 30 minutes.
The open field test is used to observe general locomotor activity and monitor the stimulant or depressant effects of pharmacological agents in rodents. An Arduino was used to detect if perpendicular infrared beams were interrupted by the mouse (a beam break; Figure 27) in a standard 5-gallon bucket. An LCD screen was connected to the Arduino to allow us to monitor the total beam breaks and running time for the experiment. Full schematics for the locomotor assay beam break recording circuit are given in Figure 27. At the end of an experiment data on the beam breaks and the time of those breaks were exported as a CSV file and analyzed using Microsoft Excel and GraphPad Prism.

**Results**

Two control mice were administered an open field test for 13 consecutive days. Mouse 1 had a higher number of beam breaks on every day of the experiment than mouse 2. It also had a larger variation in the number of steps per day (Figure 28). The average number of beam breaks per day for mouse 1 was 2406 ± 160 while the mean was 1181 ± 134 for mouse 2. Finding such a large difference between specimens was unexpected. There was no discernable trend over time in the total number of beam breaks per day. This large inter-animal variation necessitates the use of same-animal controls as much as possible.

A new group of mice were exposed to e-cigarette vapor or isolation for 30-mins. The number of beam breaks recorded every 3 minutes were binned and compared to their locomotor activity before treatment. Control treatment (no vapor) led to decreased locomotor activity at the end of the experiment (Figure 29). Animals appeared to acclimate to the locomotor chamber. Total number of beam breaks were not significantly
different between baseline and control treatment (baseline: 1948 ± 226, control: 1270 ± 295). There was no difference in e-cigarette treatment compared to baseline. Like control, the total number of beam breaks were not significantly different, but treatment with e-cigarettes increased the variability in the number of beam breaks (baseline: 1754 ± 126, e-cigarette: 1855 ± 457). E-cigarette treatment also prevented acclimation to the locomotor chamber.

**Summary**

E-cigarette exposure appears to have mild effects on mouse behavior as measured by the locomotor assay. This indicates that the Arduino-controlled exposure system works. Blood nicotine levels must be taken to validate the e-cigarette exposure system and to determine nicotine dose. However, there was a large inter-animal variation in the number of beam-breaks per day in the locomotor assay. This may be a limitation of the design of the instrument. Adding additional infrared beams to the bucket may help to reduce these differences.

Arduinos are useful devices that can be adapted to serve a variety of laboratory needs. More trials are needed to improve the accuracy and consistency of the results.
Figure 25 – Arduino-Controlled E-Cigarette Vaping Pump

Image of the vaping chamber, Arduino-controlled diaphragm pump and Vuse e-cig (A). The e-cig was “vaped” by an Arduino-activated diaphragm pump for two seconds every minute at a flow rate of 1.1 L/min. (B) Full code used to control e-cigarette pump shown in the Arduino IDE. (C) Prototype pump control circuit board connected to Arduino and pump. (D) Custom built Arduino shield to control pump.
Figure 26 - Arduino-Controlled Diaphragm Pump Circuit Diagram

Full circuit diagram from Arduino-controlled diaphragm pump.
Figure 27 - Open-Field Test Picture and Circuit Diagram

An image of the open-field test used to record locomotor activity (top). Infrared beam paths shown in red. Beam breaks were measured for 30 minutes with a 100 ms delay between beam breaks. Schematic diagram of beam break monitoring circuit (bottom).
Control Measurements of Locomotor Activity in the Open Field Test are Highly Variable

Two control mice were administered an open field test for 13 consecutive days. (A) The average number of beam breaks were remarkably different between the two animals. Bars are means ± SEMs. (B) There was no discernable trend over time in the total number of beam breaks per day.

Figure 28 - Control Measurements of Locomotor Activity in the Open Field Test are Highly Variable
Open Field Test Results Before and After Acute Exposure to E-Cigarette Vapor

**Figure 29 - Open-Field Test Results Before and After Acute Exposure to E-Cigarette Vapor**

Trace of the number of beam breaks recorded every 3 minutes (A). Control treatment (no vapor) led to decreased locomotor activity at the end of the experiment. (B) Total number of beam breaks were not significantly different between baseline and control treatment. (C) There was no difference in ECig treatment compared to baseline. (D) Similar to control, the total number of beam breaks were not significantly different. *, p < 0.05 compared to control. Data are reported as means ± SEMs. (n = 3)
Figure 30 - EFS Stimulator Design
Schematic diagram for the custom 3D-printed post designed using the TinkerCAD software (www.tinkercad.com). The stimulator post was printed using a Statasys3D printer from ABS plastic in the Reinert–Alumni Memorial Library at Creighton University. (A) Cross section of the post (D-A). Triangular indentations allow for the rail component (D-C) to be lowered into the bath. (B) Dimensions for the top cross bar that holds the post in the tissue bath (D-B). (C) Cross section of the side pieces that support the electrodes. Platinum electrodes were fixed using marine epoxy to the end of each side piece in such a manner that they could be lowered on either side of the main post. The electrode wire is threaded through the hollow interior. Triangular extensions allow for smooth sliding along the tracks in (A). (D) Shows how the cross-sectional pieces from A-C are put together. Lengths are indicated. At the bottom of the main post (D-A), a small wire was affixed with marine epoxy to support the tissue.
Bibliography


